



Terms and Conditions of Use of Digitised Theses from Trinity College Library Dublin

Copyright statement

All material supplied by Trinity College Library is protected by copyright (under the Copyright and Related Rights Act, 2000 as amended) and other relevant Intellectual Property Rights. By accessing and using a Digitised Thesis from Trinity College Library you acknowledge that all Intellectual Property Rights in any Works supplied are the sole and exclusive property of the copyright and/or other IPR holder. Specific copyright holders may not be explicitly identified. Use of materials from other sources within a thesis should not be construed as a claim over them.

A non-exclusive, non-transferable licence is hereby granted to those using or reproducing, in whole or in part, the material for valid purposes, providing the copyright owners are acknowledged using the normal conventions. Where specific permission to use material is required, this is identified and such permission must be sought from the copyright holder or agency cited.

Liability statement

By using a Digitised Thesis, I accept that Trinity College Dublin bears no legal responsibility for the accuracy, legality or comprehensiveness of materials contained within the thesis, and that Trinity College Dublin accepts no liability for indirect, consequential, or incidental, damages or losses arising from use of the thesis for whatever reason. Information located in a thesis may be subject to specific use constraints, details of which may not be explicitly described. It is the responsibility of potential and actual users to be aware of such constraints and to abide by them. By making use of material from a digitised thesis, you accept these copyright and disclaimer provisions. Where it is brought to the attention of Trinity College Library that there may be a breach of copyright or other restraint, it is the policy to withdraw or take down access to a thesis while the issue is being resolved.

Access Agreement

By using a Digitised Thesis from Trinity College Library you are bound by the following Terms & Conditions. Please read them carefully.

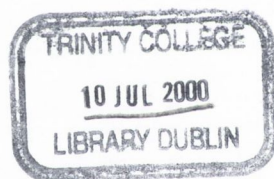
I have read and I understand the following statement: All material supplied via a Digitised Thesis from Trinity College Library is protected by copyright and other intellectual property rights, and duplication or sale of all or part of any of a thesis is not permitted, except that material may be duplicated by you for your research use or for educational purposes in electronic or print form providing the copyright owners are acknowledged using the normal conventions. You must obtain permission for any other use. Electronic or print copies may not be offered, whether for sale or otherwise to anyone. This copy has been supplied on the understanding that it is copyright material and that no quotation from the thesis may be published without proper acknowledgement.

**The role of amines
in
paraganglioma, neuroblastoma and carcinoid
diagnosis**

William P. Tormey MB, BCh, BAO, BSc (NUI), DCH, FRCPI, FFPATH RCPI.
Department of Chemical Pathology
Beaumont Hospital
Dublin 9
Ireland

Thesis submitted to Trinity College, University of Dublin, for the degree of
Doctor in Philosophy

March 2000



THESIS
5739

Index

Acknowledgements	4
Declarations	5
Summary	6

Thesis chapters

Metabolic pathway diagram	8
1. Introduction to pheochromocytoma and paraganglioma.	9
2. Milestones in the evolution of pheochromocytoma diagnosis.	15
3. Measuring catecholamines, metanephrines, HMMA, HVA and 5-HIAA in biological fluids – an outline of methods.	29
4. Laboratory methods used during the present studies including automated sequential trace enrichment of dialysates (ASTED) for sample preparation and Appendix 1 on quality assurance.	54
5. Reference values for catecholamine secretion related to urine creatinine in adults.	95
6. Results and case details in pheochromocytoma and paraganglioma.	97
7. Diagnostic sensitivities and specificities of individual catecholamines and combinations which include HMMA in the diagnosis of pheochromocytoma (Data from 1989 to 1994).	121
8. Published performances of biochemical indices in pheochromocytoma diagnosis.	123

9.	Pharmacological tests in phaeochromocytoma diagnosis.	142
10.	Catecholamine secretion and ploidy in phaeochromocytomas.	146
11.	Repetition of data in publications on DNA flow cytometry in phaeochromocytoma.	158
12.	Imaging of paragangliomas and phaeochromocytomas.	160
13.	¹²³ I-metaiodobenzylguanidine scans in phaeochromocytoma diagnosis: current clinical practice and relationship to biochemical screening.	170
14.	The excretion of catecholamines and their metabolites in patients with abnormal urinary 5-HIAA.	178
15.	Renal cell carcinoma presenting as a pseudo carcinoid tumour.	186
16.	Multiple Endocrine Neoplasia – Von Hippel Lindau – Neurofibromatosis.	189
17.	Catecholamines in urine after death.	197
18.	Clinical investigation in neuroblastoma.	203
19.	Neuroblastoma – A brief history.	218
20.	Reference values of catecholamine HMMA and HVA excretion in children related to the urine creatinine.	221
21.	Interpretation of random urinary catecholamines and their metabolites in neuroblastoma.	228
22.	Concluding discussion	236
	Publications associated with this thesis.	251

Acknowledgements

The author wishes to thank the staff of Beaumont Hospital for their co-operation, collaboration and assistance over many years. In particular Mr Roland FitzGerald has been of enormous assistance in the technical sphere. Collaboration with Dr James Finucane, Dr Shane O'Neill, Dr Marie Harte and Mr Patrick Broe on the clinical side and with Dr Elaine Kay in Histopathology for ploidy studies has been valuable. Clinicians and pathologists in the general hospitals in Sligo, Cork, Drogheda, Letterkenny, Limerick, Kilkenny, Navan and Monaghan have also co-operated constructively, have sent urine samples and discussed and sometimes referred patients. I am grateful to those pathologists who provided access to tumour tissue for the ploidy study.

Expert technical assistance in chromatography has also been provided by Ms M FitzGibbon in the early years and by Mr Michael Carney latterly.

For the study of neuroblastomas, Dr Anne O'Meara provided the clinical link and Mr Des Kenny designed the statistical presentation of the neuroblastoma data.

Dr Frank Keeling and Professor Michael Lee have been most co-operative in the discussion and subsequently reporting on appropriate imaging investigations in patients who are under investigation for possible phaeochromocytoma.

The initial gene studies have been performed in the Genetics Department at Our Lady's Hospital for Sick Children, Dublin under the direction of Professor Andrew Greene.

Ms Maria Alberici has provided organised secretarial assistance over many years and the staff of the RCSI Library at Beaumont Hospital have willingly assisted in getting access to many papers in the literature..

I would also like to acknowledge the encouragement of Dr W.G.Davis throughout my career and my late mother for the instillation of application and determination.

Declarations

The work described in this thesis was carried out by me at Beaumont Hospital, Dublin. There was close collaboration with the staff in the HPLC section of the Department of Chemical Pathology. DNA ploidy was measured in the Department of Histopathology under the guidance of Dr Elaine Kay. Dr O'Meara and Dr F. Breathnach remain the clinical contacts for neuroblastoma screening. Ms FitzGibbon wrote the initial draft of the review paper on "Paediatric reference ranges for urinary catecholamines/metabolites and their relevance in neuroblastoma diagnosis" following a systematic review of the literature with Mr Roland FitzGerald and myself. The other publications listed were written by William Tormey. Other work in the thesis is my own.

I hereby give permission to the library of Trinity College Dublin to copy this thesis in whole or in part without further reference to me.

W. P. Tormey

7 June 2000

Summary

The role of urinary noradrenaline, adrenaline, dopamine, 4-hydroxy-3-methoxy-mandelic acid (HMMA) and homovanillic acid (HVA) in the diagnosis of paraganglioma especially pheochromocytoma and in neuroblastoma is investigated. The clinical and laboratory correlates of the association of HMMA and HVA with an increased urinary 5-hydroxyindoleacetic acid (5-HIAA) were also studied. Latterly, the utility of urinary normetanephrine and metanephrine in the diagnosis of pheochromocytoma was studied. The current clinical practice and relationship of ^{123}I -metaiodobenzylguanidine scans to biochemical screening for pheochromocytoma was also studied. The relationship of 24-hour urinary catecholamines, HMMA and HVA to DNA ploidy in 14 patients with pheochromocytoma was studied. As physiological stress is common before death, catecholamines in urine after death were studied to see if there was a separation from values seen in pheochromocytoma.

Methods: The biochemical analytes were measured using HPLC with electrochemical detection. Details on drug interferences with the chromatograms are shown. Measurement of normetanephrine and adrenaline was performed using an automated sequential trace enrichment HPLC system.

Case details: there were 26 patients with sporadic pheochromocytomas, 3 with Von Hippel Lindau disease and pheochromocytoma, 3 with neurofibromatosis and pheochromocytoma, 1 with malignant metastatic paraganglioma and 1 ganglioneuroblastoma in an adult. There was no confirmed case of multiple endocrine neoplasia.

Because the excretion of catecholamines and their metabolites in children increase with age, the reference values used in neuroblastoma diagnosis were determined in a study of normal children. The interpretation of results of the screening of random urines for these analytes in the diagnosis and follow-up of neuroblastoma showed that the measurements of HMMA, HVA and dopamine will detect all but the most immature neuroblastomas. Urinary dopamine is the most reliable indicator of persistent disease. Noradrenaline and adrenaline measurements were of little benefit.

In patients where carcinoid syndrome was suspected, and the urinary 5-HIAA was greater than the reference limit, HMMA was raised in 14.3% and HVA in 21%. Flushing was the most prominent symptom in 46% and diarrhoea or altered bowel habit in 37%.

In the ploidy study, there was a significant reduction in urine noradrenaline ($p = 0.0182$) and HMMA ($p = 0.0265$) in diploid tumours but there was no difference in adrenaline, dopamine and HVA excretion using the Mann-Whitney U test. A review of published ploidy studies shows that malignancy is more than six times more common in non-diploid tumours but diploidy does not equate with benignity. No biochemical marker is a reliable index of malignancy in the individual case. There was no relationship between urinary noradrenaline excretion and tumour size.

Over an eight year period, there were no false negative MIBG scans. False positive biochemical screens were associated with MIBG scans in 35% of 42 cases where a scan was performed. 25% of the MIBG scans were performed for clinical reasons when the biochemical screen was within the reference range. MIBG is particularly useful for the detection of extra adrenal tumours and metastases.

Catecholamine values found in urine samples taken at autopsy and related to the urine creatinine overlap those found in some normal subjects and in patients with phaeochromocytoma.

The sensitivities for phaeochromocytoma diagnosis using 24-urine specimens for the period 1989 to December 1999 were noradrenaline 87.7%, adrenaline 48.1%, dopamine 20% calculated from data in 30 cases, HMMA 74.1% from data from 27 cases, and HVA 28% from data from 15 cases. The sensitivities for normetanephrine and metanephrine excretion in a retrospective analysis of stored samples from 10 cases gave sensitivities of 93.3% and 33.3% respectively. The overall sensitivity for total urinary metanephrines was 94.4% from 13 patients. The specificities for phaeochromocytoma diagnosis for the years 1989 to 1994 inclusive were 96% for noradrenaline, 93.3% for adrenaline, 96% for dopamine, 88.4% for all three catecholamines together, 95.8% for HMMA and 93.2% for all three catecholamines plus HMMA. Data from those years show the predictive value for phaeochromocytoma of a positive urinary noradrenaline was 40.9% and for urinary adrenaline was 20.7%. The predictive value of a negative urinary noradrenaline for phaeochromocytoma was 99.25% and for urinary adrenaline it was 98.3%. The diagnostic efficiency for urinary noradrenaline was 95.45%, for urinary adrenaline, it was 92%, for urinary dopamine, it was 93.3% and for urinary HMMA, it was 95.4%

Outcomes: there were six deaths from 34 cases (17.6%) due directly or indirectly to the paraganglioma. Of the 29 patients with adrenal phaeochromocytoma, 3 (10.3%) died and there have been no metastatic recurrences to date. The patient with ganglioneuroblastoma was diagnosed following autopsy.

Conclusions: A systematic protocol should be used in the investigation of phaeochromocytoma which should incorporate a 24-hour collection for urinary free catecholamines or total metanephrines to maximize sensitivity. Care must be taken in the selection of an appropriate reference range to answer the individual clinical question. A range derived from patients with hypertension should be used to screen for in the general population to minimize the number of biochemical false positives. However, a lower range is appropriate in screening those patients with family histories of von Hippel Lindau disease and multiple endocrine neoplasia type 2 to increase the diagnostic sensitivity. Annual reviews of patients post surgery should be undertaken for at least 15 years. Gene studies of the families of index patients should now be offered. Population screening for neuroblastoma is not indicated and urinary dopamine, HMMA and HVA remain the best initial biochemical screening tests.

Catecholamine formation and degradation

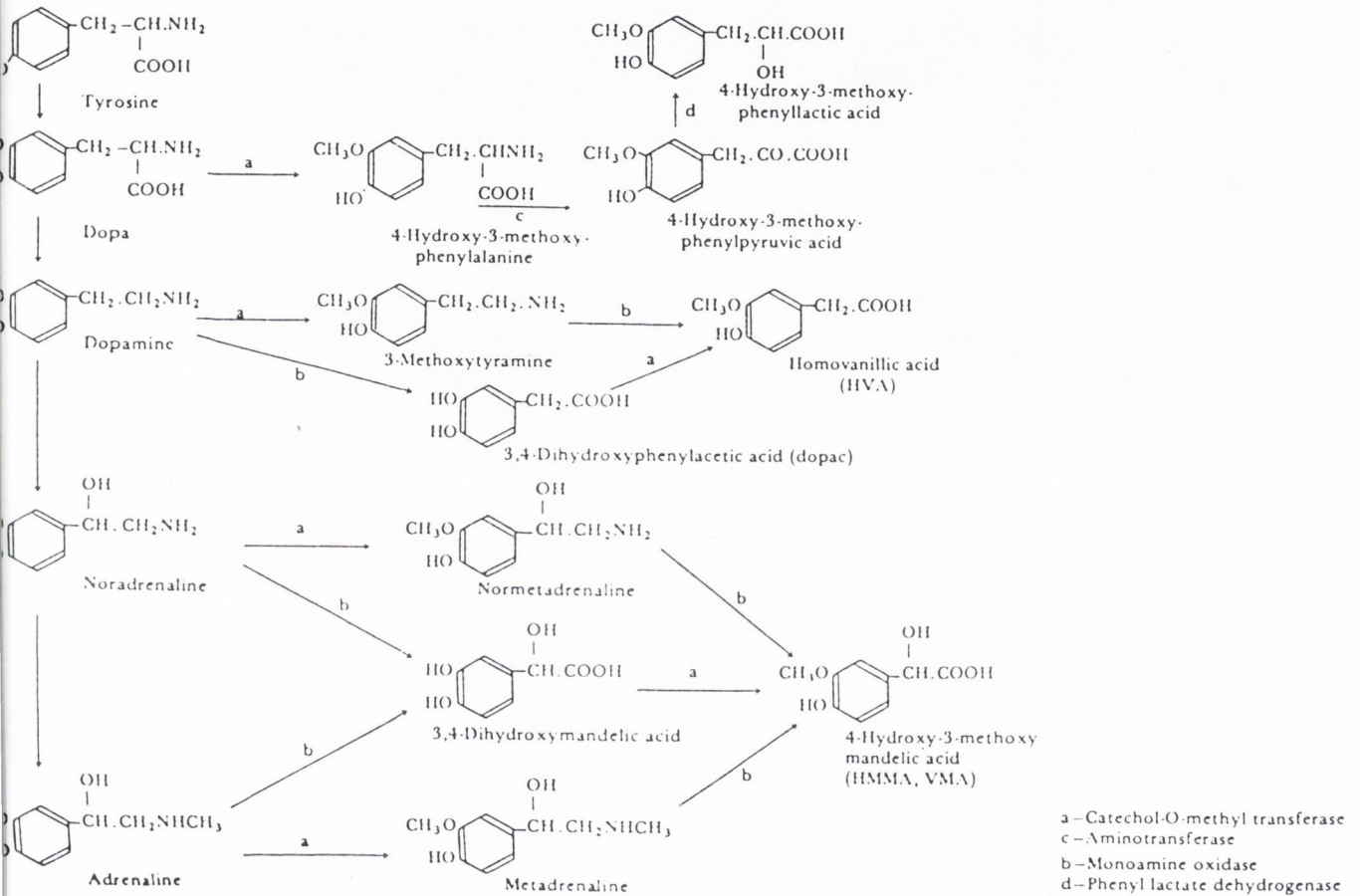


Diagram:- Varley's Practical Clinical Biochemistry, Gowenlock AH ed, 6th edn, London London Heinemann Medical books, 1988,p878.

Catecholamines are synthesized in sympathetic nerve endings, brain and chromaffin tissues. Noradrenaline and adrenaline are synthesized in chromaffin cells and brain, whereas only noradrenaline is formed in the post-ganglionic sympathetic nerves. All the enzymes necessary for the conversion of L-tyrosine to noradrenaline are present in the nerve ending. Tyrosine is derived from dietary or tissue tyrosine or from dietary phenylalanine, which is converted to tyrosine.

Introduction to phaeochromocytoma and paraganglioma

The paraganglion system is part of the dispersed neuroendocrine system and includes elements of the central and peripheral nervous systems. The paraganglion system is divided into the adrenal medulla and the extra-adrenal system. The latter is divided into the sympathetic system in the para-aortic, thoracic, and abdominal regions which is functionally related to the adrenal medulla and that related to the parasympathetic system which functions as a series of afferent receptor organs.

The extra-adrenal paraganglion system can be subdivided into (1) branchiomic, which includes the carotid, jugulotympanic, subclavian, laryngeal, coronary, aortopulmonary and orbital paraganglia; (2) intravagal paraganglia; and (3) aortic sympathetic paraganglia associated with the sympathetic system. This last group is mostly axial and extends from the aortic arch to the bladder and includes the organs of Zuckerkandl. Tumours arising from the branchiomic and intravagal paraganglia are usually chromaffin negative whereas the aortic sympathetic paraganglia are usually chromaffin positive and secrete catecholamines similar to adrenal phaeochromocytomas.

Morphologically similar paraganglionas differ widely in their clinical behaviour depending on their site of origin¹. Extra adrenal paragangliomas infrequently produce clinical symptoms related to excess hormone or catecholamine secretion, in contrast to adrenal phaeochromocytomas. Most paragangliomas are solitary and sporadic but they may be multicentric and may be familial. Extra-adrenal paragangliomas may occur as part of multiple endocrine neoplasia (MEN 2b)². Paragangliomas in the sympathoadrenal system are usually clinically benign and are cured by surgical removal. Other than overt metastases, histologic features do not allow the reliable prediction of malignant behaviour. In the past, up to 10% of adrenal paragangliomas in adults were reported to be clinically malignant³. The reported incidence of malignant behaviour of paragangliomas varies with their anatomical site. Metastatic rates are 28% - 50% for para-aortic and retroperitoneal tumours (see below), 25% for laryngeal, 21% for vagal body, 13% for aorta pulmonary, 7% for thoracic, 6% for jugulo-tympanic, 5% for urinary bladder and 2% - 9% for carotid body paragangliomas when there has been long term follow-up⁴. Malignant paragangliomas express fewer neuropeptides (an average of 2 per tumour) than benign tumours (5 per tumour)⁵.

There is a relationship between tumour site and catecholamine secretion. Excess catecholamine secretion is the norm in adrenal paragangliomas (phaeochromocytomas). Rare non-secretors have been described in a case report as recently as 1991⁶. Analysis of platelet catecholamines directly or of the difference between serum and plasma noradrenaline which is approximately equivalent to the noradrenaline content of platelets may help reduce the level of false negative results⁷. Catecholamine secretion is rare in extra adrenal paragangliomas but such tumours in the bladder^{8,9}, heart¹⁰ and para-aortic¹¹ regions have the highest incidence of catecholamine secretion.

Cell Type

The paraganglion system is composed of widely dispersed collections of specialised cells related both functionally and structurally to the adrenal medulla. Both organ systems contain two separate populations of cells derived from primitive neural precursor cells. Chief, or type I, cells are neuroectodermal in origin, contain neurosecretory granules, and may secrete catecholamines in both normal and abnormal (hyper-plastic and neoplastic) states. Sustentacular, or type II, cells closely invest clusters of chief cells, are related to satellite cells of autonomic ganglia, lack neurosecretory granules, and do not secrete or contain catecholamines. On hematoxylin-eosin and reticulin stained sections of normal organs, an organoid pattern can be seen, with type I cells most numerous, forming “zellballen” surrounded by less frequent and inconspicuous type II cells. An organoid pattern may persist in the neoplastic state. Chief cells contain neuron-specific enolase (NSE), chromogranin, met and leu-enkephalin. Sustentacular cells contain S-100 protein and glial fibrillary acid protein (GFAP)¹².

Kliwer et al¹² also state that “In adrenal medullary tissues, both the staining intensity of chief cells for chromogranin and the numbers of sustentacular cells containing S-100 protein diminish in hyperplasia and neoplasia relative to the benign state”. Sustentacular cells are either absent or markedly depleted in high grade and malignant pheochromocytomas¹³.

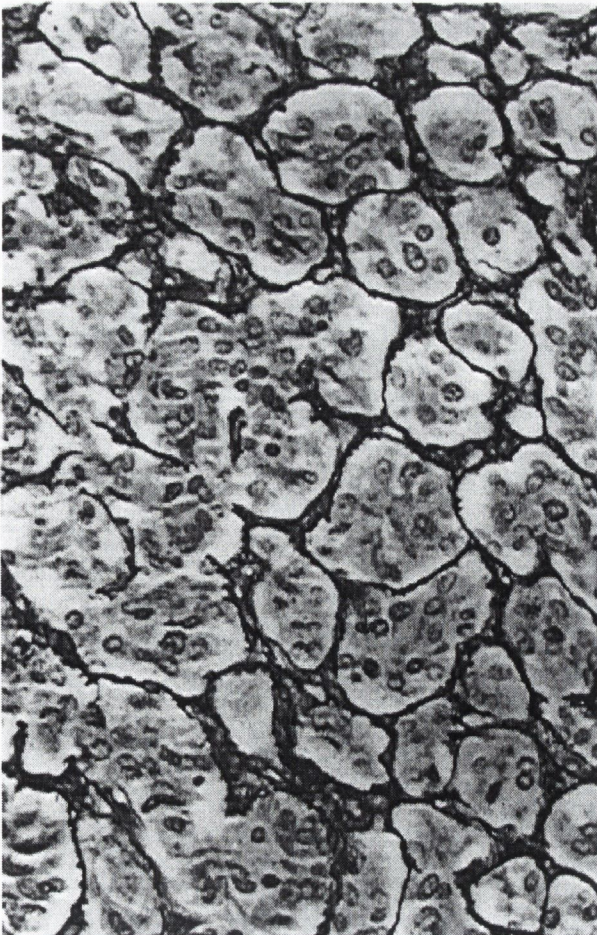


Figure 1. Stain for reticulum clearly shows the alveolar pattern with distinct nests of cells ("zellballen")

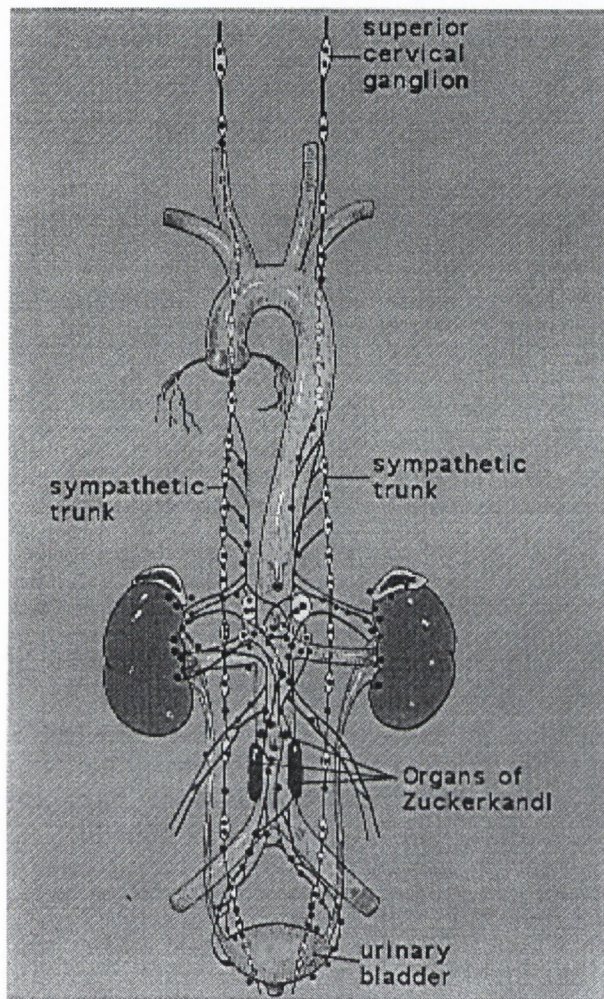
Extra-adrenal Retroperitoneal Paragangliomas

22 such patients were seen at the Memorial Sloan-Kettering Cancer Center in New York between 1949 and 1990. 8 were catecholamine secreting but this did not make a difference to the time to diagnosis, size of tumour or survival time. 50% were malignant, defined as the presence of metastases. The five and ten year disease-free survival rates were 19% and 19% for tumours not resected and 75% and 45% for those completely resected¹⁴. Reports in the literature from seven other reports of a total of 120 patients with retroperitoneal paragangliomas were summarised by Sclafini et al ¹⁴. The malignant rate varied from 22% to 50%. This contrasts with the reported 10% malignancy rate for phaeochromocytomas of the adrenal¹⁵⁻²¹.

Chemodectoma should be restricted to tumours of the carotid and aortic body receptors. These are usually non-secretory paragangliomas of a distinct anatomical site and usage may cause confusion. Carotid body tumours are about ten times more common in people living at high altitude compared to those at sea level.

It is likely that paragangliomas that are chromaffin and secrete catecholamines are usually called phaeochromocytomas when from the adrenal and extra-adrenal phaeochromocytomas when from elsewhere. Most of these arise from sympathetic-related paraganglia whereas non-functioning tumours may originate from parasympathetic-related organs. It is often impossible on morphological grounds to distinguish between these two types. Rare locations are described in Ackerman's Surgical Pathology²².

The anatomical distribution of sympathoadrenal paraganglia is shown in Figure 2 below.



(Lack EE in Tumors of the adrenal gland and extra-adrenal paraganglia in Atlas of Tumor Pathology, 3rd series, Rosai J, Sobin LH eds. Armed forces institute of Pathology, Washington DC, 1995:261.)

References

1. Grimley PM, De Lellis RA. Multi system neuroendocrine neoplasms. In: Henson DE, Albores-Saavedra J, Eds. The pathology of incipient neoplasia. Philadelphia: WB Saunders Co; 1986:425-54.
2. Larraza Hernandez O, Albores-Saavedra J, Benavides G, Krause LG, Perez-Merizaldi JC, Ginzo A. Multiple endocrine neoplasia: pituitary adenoma, multicentric papillary thyroid carcinoma, bilateral carotid body paraganglioma,

parathyroid hyperplasia, gastric leiomyoma and systemic amyloidosis. *Am J Clin Pathol*;1982;78:527-32.

3. Manger WM, Gifford RW Jr, (eds) : Pheochromocytoma. New York, Springer-Verlag, 1977.
4. Klierer KE, Cochran AJ. A review of histology, ultrastructure, immunohistology and molecular biology of extra-adrenal paragangliomas. *Arch Pathol Lab Med* 1989;113:1209-18.
5. Linnoila RI, Lack EE, Steinberg SM, Keiser HR. Decreased expression of neuropeptides in malignant paragangliomas; an immunohistochemical study. *Hum Pathol* 1988;19:41-50.
6. Sinclair D, Shenkin A, Lorimer AR. Normal catecholamine production in a patient with a paroxysmally secreting phaeochromocytoma. *Ann Clin Biochem* 1991;28:417-9.
7. Popp-Snijders C, Geenen B, van der Heijden EAP. Serum noradrenaline is composed of plasma and platelet noradrenaline. *Ann Clin Biochem* 1989;26:191-2.
8. Leestma JE, Price EB. Paraganglioma of the urinary bladder. *Cancer* 1971;28:1063-73.
9. Jurascheck F, Egloff H, Buemi A, Laedlein-Geilsammer D. Paraganglioma of urinary bladder. *Urology* 1983;22:659-63.
10. Johnson TL, Shapiro B, Beierwaltes WH, Orringer MB, Lloyd RV, Sisson JC, Thompson NW. Cardiac paragangliomas; a clinicopathologic and immunohistochemical study of four cases. *Am J Surg Pathol* 1985;9:827-34.
11. Saito H, Saito S, Sano T, Kagawa N, Hizawa K, Tatara K. Immunoreactive somatostatin in catecholamine-producing extra-adrenal paraganglioma. *Cancer* 1982;50:560-5.
12. Klierer KE, Wen D-R, Cancilla PA, Cochran AJ. Paragangliomas: assessment of prognosis by histologic, immunohistochemical and ultrastructural techniques. *Hum Pathol* 1989;20:9-39.
13. Lloyd RV, Blaivas M, Wilson BS. Distribution of chromogranin and S-100 protein in normal and abnormal adrenal medullary tissues. *Arch Pathol Lab Med* 1985;109:633-5.
14. Scalfini LM, Woodruff JM, Brennan MF. Extraadrenal retroperitoneal paragangliomas: natural history and response to treatment. *Surgery* 1990;108:1124-30.

15. Glenn F, Gray GF. Functional tumors of the organ of Zuckerkandl. *Ann Surg* 1976;183:578-86.
16. Melikow MM. One hundred cases of pheochromocytoma at the Columbia Presbyterian Medical Center 1926-1976: a clinico-pathological analysis. *Cancer* 1977;40:1987-2004.
17. Lack EE, Cubilla AL, Woodruff JM, Leiberman PH. Extra-adrenal paragangliomas of the retroperitoneum. *Am J Surg Pathol* 1980;4:109-20.
18. Scott HW, Dean RH, Oates JA, Robertson D, Rhamy RK, Page DL. Surgical management of pheochromocytoma. *Am Surg* 1981;47:8-13.
19. Van Heerden JA, Sheps SG, Hamberger B, Sheedy PF, Poston JG, ReMine WH. Pheochromocytoma: current status and changing trends. *Surgery* 1982; 91:367-73.
20. Altergott R, Barbato A, Lawrence A, Paloyan E, Freeak RJ, Prinz RA. Spectrum of catecholamine-screening tumors of the organ of Zukerkandl. *Surgery* 1985; 9: 1121-5.
21. Kryger-Beggesen N, Kjaergaard J, Sehested M. Nonchromaffin paraganglioma of the retroperitoneum. *J Urol* 1985;134:536-8.
22. Ackerman's Surgical Pathology. Juan Rosai ed, Vol 1, 7th ed, CV Mosby Co, St Louis, 1989.

Milestones in the evolution of pheochromocytoma diagnosis

- 1707 – Eustachius described the adrenal glands¹.
- 1849 – Addison told the South London Medical Society that “a diseased condition of the adrenal glands, functional or structural, may interfere with the proper elaboration of the body generally”².
- 1865 – Henle³ reported that a dark brown colour appeared in the suprarenal medullary cells when subjected to the action of chromic acid or potassium dichromate.
- 1881 – Weichselbaum described a paraganglioma at the kidney⁴.
- 1886 – Frankel associated bilateral adrenal tumours found at autopsy in an 18 year old girl with clinical episodes of palpitations, dizziness, headache and pallor of one year duration associated with a non-compressible pulse and retinopathy⁵. She also had cold sweats, episodes of chest pain and dyspnoea. She also had a goitre. Addison’s disease was the only clinical syndrome known at that time to be associated with adrenal disease and Frankel attributed the clinical features mainly to nephritis and regarded the adrenal tumours as latent. The case was recently reviewed by Welbourn⁶.
- 1887 - von Neusser reported that certain cases of hypertension were associated with tumour of the adrenal gland⁷.
- 1892 - Berdes described an adrenal medullary tumour which was likely to have been a pheochromocytoma⁸.
- 1893 - Manasse first described an adrenal pheochromocytoma⁹.
- 1895 - Oliver and Schafer¹⁰ demonstrated that adrenal medullary extracts could induce hypertension in animals.
- 1896 - Manasse demonstrated that a similar adrenal tumour stained a brownish colour when exposed to chromium salts¹¹.
- 1898 - Abel first isolated catecholamines¹².
- 1902 - Kohn showed the chromium reaction in other cells of the body¹³.
- 1903 - Kohn¹⁴ described a system of chromaffin cells lying as nests of cells in the autonomic ganglia or independently as chromaffin bodies along the entire length of the autonomic nervous system. This suggested a common embryological origin of these cells.
- 1904 - Stolz synthesised noradrenaline and noradrenaline¹⁵.

- 1904 – Marchetti reported bilateral adrenal medullary tumours¹⁶.
- 1905 – Poll¹⁷ called the tumour “phaeochromocytoma” to describe the dusky (pheo) colour (chromo) of the cut surface of the tumour when exposed to dichromate.
- 1907– Volhard may have described the clinical syndrome associated with phaeochromocytoma¹⁸.
- 1908 – Alezais and Peyron called extra adrenal chromaffin tumours arising in paraganglia “paraganglioma”¹⁹.
- 1909 – Hedinger reported another case calling it a “struma medullaris cystica suprarenalis”²⁰ and pointed out that it could be called a paraganglioma from the adrenal medulla. (Wahl in a review in 1914 wrongly attributed this as the first described example of a chromaffin tumour from the sympathetic system. Wahl HR. Neuroblastoma: with a study of a case illustrating the three types that arise from the sympathetic system. J Med Res 1914;30:205-260)
- 1910 – Barger and Dale described the pharmacological properties of noradrenaline²¹.
- 1910 – Suzuki reported a case of a paraganglioma associated with neurofibromatosis²².
- 1911 - Kawashima reported a case of a small tumour of the medulla of the adrenal gland, associated with multiple cutaneous fibromata and patchy pigmentation of the skin and believed that the cutaneous lesions bore a direct relation to the chromaffin tumour in the adrenal gland and that the case represented a disease of the sympathetic system and the adrenal gland, and that the chromaffin tumour was not an accidental complication but was a part of von Recklinghausen’s disease²³.
- 1912 - Pick suggested that adrenal tumours be called pheochromocytomas and that similar extra-adrenal tumours be called paragangliomas²⁴.
- 1913 - Helly²⁵ reported a case with hypertension and glycosuria.
- 1917 - Ogata and Ogata²⁶ thought that the chromaffin brown colour was caused by the precipitate formed by the action of adrenaline and chromium compounds.
- 1919 - Hartman and Blatz²⁷ used the chromaffin reaction to indicate the presence of adrenaline in the adrenal medulla.
- 1922 - L’Abbe, Tinel and Doumer²⁸ described the clinical syndrome caused by phaeochromocytomas.
- 1923 - Villard removed a malignant suprarenal paraganglioma from a patient who died from shock²⁹. This case is noted in Welbourn’s history⁶ but the cases of Dr Charles

Mayo and Dr Cesar Roux (below) are usually credited with being the first to successfully surgically remove a phaeochromocytoma.

- 1926 - Vaquez and Donzelot³⁰ made the first correct clinical diagnosis but the patient refused operation. The tumour was confirmed later at autopsy.
- 1926 - Roux³¹ in Lausanne, Switzerland successfully removed a right subcostal tumour which turned out to be a phaeochromocytoma.
- 1927 - Mayo³² performed the first successful surgical removal of a phaeochromocytoma in the United States from a 30 year old woman with paroxysmal hypertension of 18 months duration.
- 1929 - Pincoffs³³ reported the first correct pre-operative diagnosis with subsequent successful surgical excision of the tumour.
- 1929 - Rabin³⁴ carried out the first estimation of adrenaline in a phaeochromocytoma. He found that the tumour contained a greater amount of adrenaline than is found in normal adrenals and suggested that this was the cause of the clinical syndrome.
- 1930 - Gerard, Cordier and Lison³⁵ showed that the brown colour could be produced by mixing adrenaline with other strong oxidizing compounds. The chromaffin reaction was an oxidizing reaction and not simply an index of chromium affinity.
- 1932 - Eisenberg and Wallerstein described the association of phaeochromocytoma and thyroid carcinoma in a 63 year old woman³⁶.
- 1934 - Wolf and Wilens reported a case of Lindau's disease and paraganglioma³⁷.
- 1936 - Rosenthal and Willis³⁸ reviewed the association of paraganglioma associated with neurofibromatosis.
- 1937 - Beer, King and Prinzmetal³⁹ showed the presence of a pressor substance in the blood of a patient with a hypertensive crisis from phaeochromocytoma with systolic pressure >300 mmHg. Using a modification of the Pissemiski method of perfusion of a denervated rabbit's ear as a test organ, infused patient's plasma showed a marked pressor effect when compared to plasma from a control subject.
- 1937 - Blaschko, Richter and Schlossmann reported the oxidative deamination pathway of catecholamine metabolism⁴⁰.
- 1939 - Blaschko proposed the synthesis of adrenaline from phenylalanine through tyrosine, dihydroxyphenylalanine, dopamine and noradrenaline⁴¹.
- 1939 - Strombeck and Hedberg using a chemical assay showed an increased blood adrenaline in a patient with this tumour⁴².

- 1942 – Pheochromocytoma could produce sustained hypertension⁴³.
- 1943 – Hyman and Mencher first described familial pheochromocytoma⁴⁴.
- 1945 - Roth and Kvale⁴⁵ introduced the histamine provocation test as the first pharmacological test to improve the accuracy of diagnosis.
- 1946 - von Euler⁴⁶ first demonstrated the presence of noradrenaline in extracts of post-ganglionic adrenergic nerves of cattle.
- 1947 - Holtz, Credner and Kroneberg⁴⁷ showed that the adrenal medulla contained some noradrenaline as well as adrenaline.
- 1947 - Calkins and Howard again reported the familial occurrence of pheochromocytoma⁴⁸.
- 1947 - Goldenberg, Snyder and Aranow showed that an adrenergic blocking agent, benzodioxan dramatically lowered the blood pressure in a patient with pheochromocytoma⁴⁹.
- 1949 - Holton⁵⁰ in England and Goldenberg, Faber, Alston and Chargaff⁵¹ in the United States reported that large amounts of noradrenaline and adrenaline were present in pheochromocytomas.
- 1949 - Grimson⁵² introduced the phentolamine (regitine) (α -blocker) test.
- 1949 - Bulbring reported the conversion of noradrenaline to adrenaline by the adrenal gland⁵³.
- 1950 - Quantitation of pressor amines in blood⁵⁴.
- 1951 – About 150 cases had been surgically treated. Graham⁵⁵ reported on 125 of these: 33 (26%) had died and 92 had had a successful surgical excision.
- 1951 - Von Euler reported an increase in urine noradrenaline and adrenaline excretion in pheochromocytoma⁵⁶.
- 1951 - Iseri, Henderson and Derr reported the efficacy of phentolamine in medical management⁵⁷.
- 1952 - De Courcy and De Courcy wrote “a significantly high percentage of patients with pheochromocytoma have had coexistent diffuse or nodular goitre or thyroid carcinoma”⁵⁸.
- 1952 - Quantitation of urinary catecholamines lead to increased accuracy of diagnosis⁵⁹.

- 1953 – Glushien et al reported the association of neurofibromatosis, Von Hippel-Lindau and phaeochromocytoma⁶⁰.
- 1954 – Wermer noted the genetic aspects of adenomatosis of the endocrine glands in a single family with tumours of the pituitary, parathyroid and pancreatic islet cells⁶¹.
- 1954 – Goldenberg et al published the results of screening of urine for adrenaline and noradrenaline in 16 patients with phaeochromocytoma⁶².
- 1954 – Manger et al reported the results of fluorometric quantitation of plasma adrenaline and noradrenaline in 13 patients with phaeochromocytoma. They also pointed out the potential use of histamine administration as a provocative test⁶³. They demonstrated the rare occurrence of dopamine in an extract of pheochromocytoma.
- 1957 – Armstrong, MacMillan and Shaw identified 3-methoxy-4-hydroxymandelic acid as a major metabolite of adrenaline and noradrenaline in urine⁶⁴.
- 1957 - Shaw, McMillan and Armstrong showed the presence of homovanillic acid (3-methoxy-4-hydroxyphenylacetic acid) in normal human urine⁶⁵.
- 1958 - LaBrosse et al reported increased urine concentrations of HMMA and normetanephrine in phaeochromocytoma⁶⁶.
- 1958 - Axelrod et al⁶⁷ and LaBrosse et al⁶⁸ established that O methylation was the principal pathway for the metabolism of these catecholamines in the blood and some tissues.
- 1960 - Gitlow et al reported the diagnosis of phaeochromocytoma by assay of HMMA⁶⁹.
- 1961 - Sipple described the association of phaeochromocytoma with thyroid carcinoma⁷⁰.
- 1961 - Crout, Pisano and Sjoerdsma reported on the excretion of catecholamines and their metabolites in phaeochromocytoma⁷¹.
- 1962 – Chapman and Diaz-Perez described the association of cerebellar haemangioblastoma and phaeochromocytoma⁷².
- 1962 - Tisherman et al noted the familial association of phaeochromocytoma with von Hippel Lindau disease traced through five generations⁷³.
- 1962 - Cushman reported that parathyroid adenomas are associated with phaeochromocytoma and thyroid carcinoma⁷⁴.
- 1963 - Manning et al also reported the finding of phaeochromocytoma, thyroid carcinoma and parathyroid adenomas occurring together⁷⁵.

- 1967 - Chromogranin A was purified and characterised from catecholamine storage vesicles of the bovine adrenal medulla^{76,77}.
- 1968 - Steiner et al⁷⁸ called the coexistence of pheochromocytoma, medullary carcinoma of the thyroid and parathyroid tumour – Multiple Endocrine Adenomatosis Type 2 and called the entity of tumours of the pituitary, parathyroids and pancreas – Multiple Endocrine Adenomatosis Type 1.
- 1968 - Schimke et al described the syndrome of pheochromocytoma, medullary thyroid carcinoma and multiple neuromas⁷⁹.
- 1972 - Goldstein showed two distinct cell types in the adrenal medulla, one containing noradrenaline, the other adrenaline⁸⁰.
- 1974 - ACTH found in pheochromocytoma⁸¹.
- 1975 - Multiple Endocrine Neoplasia Type 2b – ganglioneuromatosis, marfanoid habitus, medullary thyroid carcinoma, pheochromocytoma and rarely parathyroid hyperplasia⁸².
- 1975 - Vasoactive intestinal peptide (VIP) found in pheochromocytoma⁸³.
- 1978 – Hypercalcaemia found in half of pheochromocytomas tested⁸⁴.
- 1978 – Met-enkephalin shown in pheochromocytoma⁸⁵.
- 1978 – Localization of pheochromocytomas shown using computed tomography⁸⁶.
- 1979 - Lundberg et al showed somatostatin-like immunoreactivity in pheochromocytoma⁸⁷.
- 1981 – Sisson et al first localised pheochromocytomas using ¹³¹I MIBG⁸⁸.
- 1983 – Imaging of adrenal gland by nuclear magnetic resonance imaging⁸⁹.
- 1984 - Chromogranin A was purified from the catecholamine-storage vesicles of pheochromocytoma⁹⁰.
- 1984 - Oxytocin and arginine vasopressin shown in pheochromocytoma tissue⁹¹.
- 1987 - Gene causing MEN 2 mapped to the centromeric region of chromosome 10^{92,93}.
- 1988 - Gene causing MEN 1 mapped to pericentromeric region of the long arm of chromosome 11, band 11q13⁹⁴.
- 1991 - MEN 2a, MEN 2b and familial medullary thyroid carcinoma map to the same region on chromosome 10⁹⁵.

- 1993 – Ret proto-oncogene mutations on chromosome 10 shown as the cause of MEN Type 2⁹⁶.
- 1993 - The von Hippel-Lindau gene was mapped to chromosome 3p25.3⁹⁷ and identified by positional cloning⁹⁸.
- 1994 - Allelic loss in the neurofibromin gene was shown to be associated with neurofibromatosis type I associated pheochromocytomas⁹⁹.
- 1995 - Mutations in codon 238 were identified in more than 40% of von Hippel-Lindau families with pheochromocytoma¹⁰⁰.
- 1995 - Loss of neurofibromatosis type 1 gene expression was also shown in some pheochromocytomas from patients with neurofibromatosis type 1¹⁰¹.

References

1. Eustachius B. Opuscula anatomica de renum structura, Lugduni Batavorum, apud J van der Linden. Chap 6, p15 (De glandulis que renibus incumbant).
2. Cardiovascular Pathology ed REB Hudson Vol 3. Ed Arnold Ltd. London 1970,848.
3. Henle J. Ueber das gewebe der nebenniere und der hypophyse. Ztsche f rat med, Leipz u Heidelb. 1865;3:R,xxiv;143-152.
4. Weichselbaum Arch f Virchow 1881;lxxxv:554.
5. Fraenkel F. Ein fall von doppelseitigem, vollig latent verlaufenen nebennierentumor und gleichzeitiger nephritis mit veränderungen am circulationsapparat und retinitis. Virchows Arch f Path Anat Physiol und f Klin Med 1886;103:244-263.
6. Welbourn RB. Early surgical history of pheochromocytoma. Br J Surg 1987;74:594- 6.
7. Von Neusser. Die erkrankungen der nebenneiren. Ed 2, Vienna, 1910 Alfred Holder, p 97.
8. Berdes. Contribution a l'étude des tumeurs des capsules surrenales. Archives de medicine experimentale et d'anatomie pathologique. 1892;4:412-5.
9. Manasse P. Ueber die hyperplastischen tumoren der nebennieren. Virchows Arch f Path Anat 1893;133:391-404.
10. Oliver G, Schafer EA. The physiological effects of extracts of the suprarenal capsules. J Physiol 1895;18:230.

11. Manasse P. Zur histologie and histogenese der primaren nierengeschwulste. Virchows Arch Pathol Anat 1896;145:113-57.
12. Abel JJ. On epinephrin, the active constituent of the suprarenal capsule and its compounds. Proc Am Physiol Soc Boston 1898-9.piii. and also Further observations on the chemical nature of the active principle of the suprarenal capsule. Johns Hopkins Hosp Bull Baltimore 1898;ix:215-8.
13. Kohn A. Das chromaffine Gewebe. Z Anat Entwicklungsgesch 1902;12:253.
14. Kohn A. Die paraganglien. Arch mikr Anat 1903;62:263.
15. Stolz F. Ueber adrenalin und alkylaminoacetobenzcatechin. Ber Dtsch Chem Gest 1904;37:4149.
16. Marchetti G. Beitrag zur Kenntniss der pathologischen Anatomie der Nebennieren. Virchows Arch Pathol Anat 1904;177:227-48.
17. Poll H. Die vergleichende entwicklung der nebennier-ensysteme, in Hertwig O (ed): Handbuch der entwicklungsgeschichte des menschen und der wirbeltiere, vol 3. Jena, Gustav Fischer, 1905,443.
18. Volhard F. Die doppelseitigen hamatogenen Nirenerkrankungen Handbuch d inn Med v Bergmann-Stahelin. Vol VI. Berlin, J Springer, 1931.
19. Alezais and Peyron: Un groupe nouveau de teumeurs epitheliales: les paraganglions. C R Seances Soc Biol Paris 1908;65:745-7.
20. Hedinger E. Struma medullaris cystica suprarenalis. (Beetrag zur lehreder paragangliome) Frankfurter Zeitschr f Path. Festschr Wiesb.1911;vii:112-26.
21. Barger G, Dale HH. Chemical structure and sympathomimetic action of amines. J Physiol 1910;41:19
22. Suzuki S. Ueber zwei tumoren aus nebennierenmarkgewebe. Berl Klin Wchnschr 1910;47:1623-5.
23. Kawashima. Arch f Virchow 1911;cciii.
24. Pick L. Das ganglioma embryonale sympathicum (Sympathoma embryonale). Berl Klin Wchnschr 1912;49:16-22.
25. Helly C. Zeit pathologie der nebenniere. Munchen Med Wchnschr 1913;60:1811-2.
26. Ogata T, Ogata A. Henle's reaction of the chromaffin cells in the adrenals, and the microscopic test for adrenalin. J Exp Med Balt. 1917;xxv:807-17. And later translated

and republished as *Über die Helle'sche chromreaktion der sogenannten chromaffinen Zellen und den mikrochemischen nachweis des adrenalins*. Beitr z path anat u z allg path. Jena 1922-23;71:376-87.

27. Hartman FA, Blatz WE. Death produced by tying the adrenal veins. *Endocrinology* 1919;3:137-44.
28. L'Abbe M, Tinel J, Doumer E. Crises solaires et hypertension paroxystique en rapport avec une tumeur surrenale. *Bull et Mem Soc Med d. Hop de Paris* 1922;46:982-90.
29. Masson P, Martin JF. Paragangliome surrenale, etude d'un cas humain de tumeurs malignes de la medullo-surrenale. *Bull de l'Assoc francais pour etude du cancer* 1923;12:135-43.
30. Vaquez H, Donzelot E. Les crises d'hypertension arterielle paroxystique. *Presse Med* 1926;34:1329-31.
31. Van Heerden JA. First encounter with pheochromocytoma. *Am J Surg* 1982;144:277-9.
32. Mayo CH. Paroxymal hypertension with tumor of retroperitoneal nerve. Report of a case. *JAMA* 1927;89:1047-50.
33. Pincoffs MC. A case of paroxysmal hypertension associated with suprarenal tumor. *Tr A Am Physicians* 1929;44:295-9.
34. Rabin CB, Chromaffin cell tumor of the suprarenal medualla (pheochromocytoma). *Arch Path* 1929;44:228-43.
35. Gerard P, Cordier R, Lison L. Sur la nature de la reaction chromaffine. *Bull d'histol appliq a la physiol* 1930;7:133-9.
36. Eisenberg AA, Wallerstein H. Phaeochromocytoma of suprarenal medulla (paraganglioma); clinico-pathologic study. *Arch Path* 1932;14:818-36.
37. Wolf A, Wilens SL. Multiple hemangioblastomas of the spinal cord with syringomyelia: a case of Lindau's disease. *Am J Path* 1934;10:545
38. Rosenthal DB, Willis RA. The association of chromaffin tumours with neurofibromatosis. *J Path Bact* 1936;42:599-603.
39. Beer E, King FH, Prinzmetal M. Pheochromocytoma with demonstration of pressor (adrenalin) substance in the blood preoperatively during hypertensive crises. *Ann Surg* 1937;106:85-91.

40. Blaschko H, Richter D, Schlossmann H. The oxidation of adrenaline and other amines. *Biochem J* 1937;31:2187-96.
41. Blaschko H. The specific action of L-dopa decarboxylase. *J Physiol (Lond)* 1939;96:50p-51p.
42. Strombeck JP, Hedberg TP. Tumor of the suprarenal medulla associated with paroxysmal hypertension. Report of a case preoperatively diagnosed and cured by extirpation after capsular incision. *Acta Chir Scand* 1939;82:177-89.
43. Kirshbaum JD, Balkin RB. Adrenaline-producing pheochromocytoma of the adrenal associated with hypertension: report of three cases. *Ann Surg* 1942;116:54
44. Hyman A, Mencher WH. Pheochromocytoma of the adrenal gland. *J Urol* 1943;49:755-71.
45. Roth GM, Kvale WF. A tentative test for pheochromocytoma. *Am J Med Sci* 1945;210:653-60.
46. Von Euler US. Sympathin in adrenergic nerve fibres. *J Physiol* 1947;105:26p.
47. Holtz P, Credner K, Kroneberg G. Über das sympathicomimetische pressorische prinzip des harns ("Urosympathin"). *Arch f. exper. Path u Pharmacol* 1947;204:228-43.
48. Catkins E, Howard JE. Bilateral familial phaeochromocytoma with paroxysmal hypertension, successful surgical removal of tumors in two cases, with discussion of certain diagnostic procedures and physiological considerations. *J Clin Endocrinol* 1947;7:475.
49. Goldenberg M, Snyder CH, Aranow H Jr. New test for hypertension due to circulating epinephrine. *JAMA* 1947;135:971-6.
50. Holton P. Noradrenalin in tumours of the adrenal medulla. *J Physiol* 1949;108:525-9.
51. Goldenberg M, Faber M, Alston EJ, Chargaff EC. Evidence for the occurrence of nor-epinephrine in the adrenal medulla. *Science* 1949;109:534-5.
52. Grimson KS, Longino FH, Kernodle CE, O'Rear HB. Treatment of a patient with a pheochromocytoma: use of an adrenolytic drug before and during operation. *JAMA* 1949;140:1273-4.
53. Bulbring E. *Br J Pharmac Chemother* 1949;4:234-44.
54. Lund A. Simultaneous fluorimetric determination of adrenaline and noradrenaline in blood. *Acta Pharmacol et Toxicol* 1950;6:137-46.

55. Graham JB. Pheochromocytoma and hypertension; an analysis of 207 cases. *Internat abstr Surg* 1951;92:105-21.
56. von Euler US. Increased urinary excretion of noradrenaline and adrenaline in cases of pheochromocytoma. *Ann Surg* 1951;134:929-33.
57. Iseri LT, Henderson HW, Derr JW. Use of adrenolytic drug, regitine, in pheochromocytoma. *Am Heart J* 1951;42:129-36.
58. DeCourcy JL, DeCourcy CB. Pheochromocytoma and the general practitioner, Cincinnati, 1952. Barclay Newman.p90.
59. Lund A. Adrenaline and noradrenaline in blood and urine in cases of pheochromocytoma. *Scand J Clin Lab Invest* 1952;4:263-5.
60. Glushien AS, Mansuy MM, Littman DS. Pheochromocytoma. Its relationship to the neurocutaneous syndromes. *Am J Med* 1953;14:318-27.
61. Wermer P. Genetic aspects of adenomatosis of endocrine glands. *Am J Med* 1954;15:363-71.
62. Goldenberg M, Serlin I, Edwards T, Rapport MM. Chemical screening methods for the diagnosis of pheochromocytoma. I-norepinephrine and epinephrine in human urine. *Am J Med* 1954;16:310-27.
63. Manger WM, Flock EV, Berkson J, Bollman JL, Roth GM, Blades EJ, Jacobs M. Chemical quantitation of epinephrine and norepinephrine in thirteen patients with pheochromocytoma. *Circulation* 1954;10:641-52.
64. Armstrong MD, McMillan A, Shaw KNF. 3-methoxy-4-hydroxymandelic acid, a urinary metabolite of norepinephrine. *Biochem Biophys Acta* 1957;25:422-3.
65. Shaw KNF, McMillan A, Armstrong MD. *J Biol Chem* 1957;226:255-66.
66. LaBrosse EH, Axelrod J, Sjoerdsma A. urinary excretion of normetanephrine by man. *Fed Proc* 1958;17:386.
67. Axelrod J, Inscoe JK, Senoh S, Witkip B. O-Methylation, the principal pathway for the metabolism of epinephrine and norepinephrine in the rat. *Biochim-Biophys Acta* 1958;27:210-1.
68. LaBrosse EH, Axelrod J, Kety SS. O-methylation, the principal route of metabolism of epinephrine in man. *Science* 1958;128:593-4.

69. Gitlow SE, Mendlowitz M, Khassis S, Cohen G, Sha J. The diagnosis of pheochromocytoma by determination of urinary 3-methoxy-4-hydroxymandelic acid. *J Clin Invest* 1960;39:221-226.
70. Sipple JH. Association of pheochromocytoma with carcinoma of thyroid gland. *Am J Med* 1961;31:163-6.
71. Crout JR, Pisano JJ, Sjoerdsma A. Urinary excretion of catecholamines and their metabolites in pheochromocytoma. *Am Heart J* 1961;61:375-81.
72. Chapman RC, Diaz-Perez R. Pheochromocytoma associated with cerebellar hemangioblastoma, familial occurrence. *JAMA* 1962;182:1014-7.
73. Tisherman SE, Gregg FJ, Danowski TS. Familial pheochromocytoma. *JAMA* 1962;182:152-4.
74. Cushman P. Familial endocrine tumors. *Am J Med* 1962;32:352-60.
75. Manning PC Jr, Molnar GD, Black BM, Priestley JT, Woolner LB. Pheochromocytoma, hyperparathyroidism and thyroid carcinoma occurring coincidentally. *N Engl J Med* 1963;268:68-72.
76. Smith AD, Winkler H. Purification and properties of an acidic protein from chromaffin granules of bovine adrenal medulla. *Biochem J.* 1967;103:483-92.
77. Smith WJ, Kirshner N. A specific soluble protein from the catecholamine storage vesicles of bovine adrenal medulla. 1. Purification and chemical characterization. *Mol Pharmacol* 1967;3:52-62.
78. Steiner AL, Goodman AD, Powers SR. Study of a kindred with pheochromocytoma, medullary thyroid carcinoma, hyperparathyroidism and Cushing's disease: multiple endocrine neoplasia, type 2. *Medicine* 1968;47:371-409.
79. Schimke RN, Hartmann WH, Prout TE, Rimoin DL. Syndrome of bilateral pheochromocytoma, medullary thyroid carcinoma and multiple neuromas. *N Engl J Med* 1968;279:1-6.
80. Goldstein M, Fuxe K, Hokfelt T. Characterization and tissue localization of catecholamine synthesizing enzymes. *Pharmacol Rev* 1972;24:293-309.
81. Amatruda TT, Upton GV. Hyperadrenocorticalism and ACTH-releasing factor. *Ann NY Acad Sci* 1974;230:168.
82. Chong GC, Beahrs OH, Sizemore GW, Woolner LH. Medullary carcinoma of the thyroid gland. *Cancer* 1975;35:695-704.

83. Said SI, Faloona GR. Elevated plasma and tissue levels of vasoactive intestinal polypeptide in the watery-diarrhea syndrome due to pancreatic bronchogenic and other tumors. *N Engl J Med* 1975;293:155-60.
84. Raue F, Bayer JM, Rahn KH, Herfath C, Minne H, Ziegler R. Hypercalcitoninemia in patients with pheochromocytoma. *Klin Wochenschr* 1978;56:697-701.
85. Sullivan SN, Bloom SR, Polak JM. Enkephalin in peripheral neuroendocrine tumours. *Lancet* 1978;I:986-7.
86. Stewart BH, Bravo EL, Haaga J, Meaney TF, Tarazi R. Localization of pheochromocytoma by computed tomography. *N Engl J Med* 1978;229:460-1.
87. Lundberg TM, Hamberger B, Schultzberg M, Hokfelt T, Granberg P-O, Efendic S, Terenius L, Goldstein M, Luft R. Enkephalin- and somatostatin-like immunoreactivities in human adrenal medulla and pheochromocytoma. *Proc Nat Acad Sci USA* 1979;76:1007-9
88. Sisson JC, Frager MS, Valk TW, Gross MD, Swanson DP, Wieland DM, Tobes MC, Beierwaltes WH, Thompson NW. Scintigraphic localization of pheochromocytoma. *N Engl J Med* 1981;305:12-17.
89. Moon KL, Hricak H, Crooks LE et al NMR imaging of the adrenal gland: a preliminary report. *Radiology* 1983;147:155-60.
90. O'Connor DT, Frigon RP, Sokoloff RL. Human chromogranin A: purification and characterization from catecholamine storage vesicles of human pheochromocytoma. *Hypertension* 1984;6:2-12.
91. Ang VTY, Jenkins JS. Neurohypophysial hormones in the adrenal medulla. *J Clin Endocrinol Metab* 1984;58:688-91.
92. Matthew CGP, Chin KS, Easton DF, Thorpe K, Carter C, Lion GI, Fong SL, Bridges CDB, Haak H, Kruseman ACH, Schifter S, Hansen HH, Telenius H, Telenius-Berg M, Ponder BAJ. A linked genetic marker for multiple endocrine neoplasia type 2A on chromosome 10. *Nature* 1987;328:527-8.
93. Simpson NE, Kidd KK, Goodfellow PJ et al. Assignment of multiple endocrine neoplasia type 2A to chromosome 10 by genetic linkage, *Nature* 1987;328:528-30.
94. Larson C, Skogseid B, Oberg K, Nakamura Y, Nordenskjold MC. Multiple endocrine neoplasia type 1 gene maps to chromosome 11 and is lost in insulinoma. *Nature* 1988;332:85-7.
95. Lairmore TC, Howe JR, Korte JA, Dilley WG, Aine L, Aine E, Wells SA Jr, Donis-Keller H, et al Familial medullary thyroid carcinoma and multiple endocrine neoplasia

- type 2B map to the same region of chromosome 10 as multiple endocrine neoplasia type 2A. *Genomics* 1991;9:181-92.
96. Mulligan LM, Kwok JBJ, Healey CS, Elsdon MJ, Eng C, Gardner E, Love DR, Mole SE, Moore JK, Papi L et al Germ-line mutations of the RET proto-oncogene in multiple endocrine neoplasia type 2A. *Nature* 1993;363:458-60.
 97. LaForgia S, Lasota J, Latif F, Boghosian-Sell L, Kastury K, Ohta M, Druck T, Atchison L, Cannizzaro LA, Berne G et al, Detailed genetic and physical map of the 3p chromosome region surrounding the familial renal cell carcinoma chromosome translocation, t(3; 8) (p14.2; q24.1) *Cancer Res* 1993;53:3118-24.
 98. Latif F, Tory K, Gnarr J, Yao M, Duh FM, Orcutt ML, Stackhouse T, Kuzmin I, Modi W, Geil L et al. Identification of the von Hippel-Lindau disease tumor suppressor gene. *Science* 1993;260:1317-20.
 99. Gutmann DH, Cole JL, Stone WJ, Ponder BA, Collins FS. Loss of neurofibromin in adrenal gland tumors from patients with neurofibromatosis type 1. *Genes Chromosomes Cancer* 1994;10:55-8.
 100. Chen F, Kishida T, Yao M, Hustad T, Glavic D, Dean M, Gnarr JR, Orcutt ML, Duh FM, Glenn G et al. Germline mutations in the von Hippel-Lindau disease tumor suppressor gene: correlations with phenotype. *Human Mutat* 1995;5:66-75.
 101. Gutmann DH, Geist RT, Rose K, Wallin G, Moley JF. Loss of neurofibromatosis type 1 (HFI) gene expression in pheochromocytomas from patients without NF1. *Genes Chromosomes Cancer* 1995;13:104-9.

Measuring catecholamines, metanephrines, HMMA, HVA and 5-HIAA in biological fluids – an outline of methods.

- (a) Biological
- (b) Chemical using fluorimetry and spectrophotometry
- (c) Radioenzymatic
- (d) Chromatography
 - Thin layer and paper chromatography
 - Column chromatography
 - Gas chromatography
 - High performance liquid chromatography (HPLC) with fluorescence and electrochemical detection
- (e) Immunoassay
- (f) Capillary electrophoresis
- (g) Chemiluminescence

Introductory physiology

Catecholamines are transported in blood unbound to proteins and have a short plasma half-life of about two minutes. Noradrenaline is the major circulating unconjugated catecholamine and the plasma level represents a small fraction of the exocytosed neurotransmitter released from storage granules that escapes re-uptake by the post-ganglionic sympathetic nerves. Noradrenaline is inactivated by re-uptake into storage at the nerve endings, or by conversion to metabolites or by excretion in the urine as free amines or conjugates of glucuronide and sulphates. The plasma concentration is normally about 500ng/L. Unconjugated adrenaline is present in plasma almost exclusively due to release from the adrenal medulla. Plasma dopamine circulates primarily conjugated to sulphate. For plasma and urine measurements, the unconjugated fraction of catecholamines is least affected by diet. Urinary unconjugated noradrenaline and adrenaline originate from plasma clearance of the adrenergic system. But urine dopamine originates primarily from peripheral metabolism of 3,4-dihydroxy-phenylalanine (DOPA) in the kidney. Urine dopamine is the major unconjugated catecholamine in urine¹. Urine 4-hydroxy-3-methoxy-mandelic acid (HMMA) represents about 40% to 50% of the urinary excretion production of noradrenaline and adrenaline. Noradrenaline is the major source of HMMA with metabolism through MHPG as the major pathway². As a result of active neuronal re-uptake and deamination of noradrenaline, normetanephrine normally represents <5% of the total excretion products of noradrenaline in urine. Metanephrine is a major excretion product of adrenaline and is found at a lower urine concentration than normetanephrine.

Standardized conditions are necessary to validly use plasma catecholamines in diagnosis. Blood should be collected from a fasting subject who has been resting in the supine position for 20/30 minutes following the insertion of a venous catheter.

Catecholamines are generally unstable compounds and can be readily oxidized. To prevent this oxidation, many procedures recommend that blood samples be transported to the laboratory on ice, centrifuged at 4°C within 30 minutes of collection, and the plasma

removed and frozen at -70°C until analysis. Two anticoagulants are commonly used, heparin and ethylenediamine-metabisulphite. Advocates of a more liberal and less constrained method of collecting and processing blood catecholamines recommend the use of heparin without added preservatives. Catecholamine values from heparinized blood appear to be quite stable both before separation (6h at 24°C) and after separation (24 h at 24°C). At -70°C , catecholamines in heparin or EDTA plasma are stable for 8 months without the addition of preservatives³.

(a) Biological Methods

These methods have been replaced by chemical methods but bioassays can be used in pharmacology to assay catecholamines.

Cat Nictitating Membrane

This method was devised more than 60 years ago and can be used to measure adrenergic interactions⁴.

Isolated Guinea Pig Heart

This can be used to assay noradrenaline and adrenaline in plasma⁵. The method was considered highly qualitative and only semi-quantitative.

Rat Aorta

This tissue method is not specific for catecholamines as vascular smooth muscle will react to many other amines and polypeptides⁶.

Central artery of the rabbit's ear

This method is sensitive enough for catecholamines⁷. The artery is removed from the ear, cannulated and infused with Kreb's or McEwan's solution. A roller pump supplies the perfusion pressure at a constant rate and this is recorded with a mercury manometer. The artery is suspended in a bath at 37°C . Injections of adrenaline or noradrenaline cause the perfusion pressure to rise and the increments can be measured. 5-Hydroxytryptamine can potentiate the effects of noradrenaline and adrenaline in this system.

Superfused rat stomach strip in series with a chick rectum

The chick rectum is about 100 times more sensitive to adrenaline than to noradrenaline. By comparing the reactions of the two tissues to an unknown mixture with the reactions to adrenaline and noradrenaline, the proportions of each in solution can be calculated⁸.

Isolated rat uterus pretreated with stilboestrol

This is a method of discriminating between adrenaline and noradrenaline by an action of catecholamines on beta receptors. The uterus contracts in response to carbachol or 5-hydroxytryptamine and adrenaline inhibits the contraction in a concentration dependent manner. Noradrenaline is about 10,000 to 1,000,000 times less potent than adrenaline⁹.

Pithed rat

Use a transducer and manometer to record changes in blood pressure in response to applied pressor substances¹⁰.

Blood bath organ technique

Superfusion of organs with blood from an anaesthetised animal is used. Changes in catecholamine levels are measured by using the rat stomach strip and chick rectum as a detector. With the blood bath technique, it is possible to estimate the release, distribution and uptake of catecholamines directly and continuously without purifying them from samples of blood¹¹.

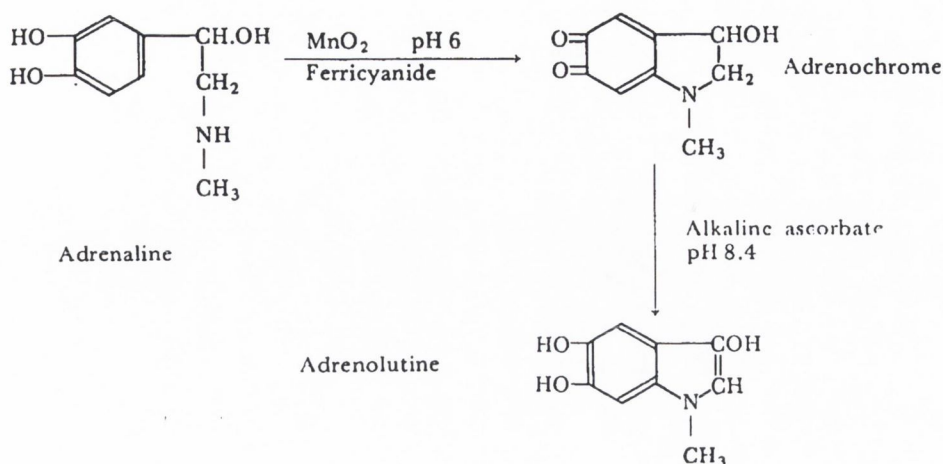
Tissue catecholamines

In 1949, Von Euler and Hamberg¹² used the coloured 2-iodo derivatives of the reaction of iodine with adrenochromes to determine catecholamines in tissues.

Methods for the estimation of plasma catecholamines

(i) Fluorometric chemical assays

In the trihydroxyindole (THI) method, oxidation converts adrenaline into adrenochrome and in alkaline solution this changes into adrenolutine, the trihydroxyindole reaction. Adrenolutine has a yellowish-green fluorescence which however disappears rapidly in presence of oxygen. Noradrenaline is converted into similar compounds, noradrenochrome and noradrenolutine^{13,14}. Manganese dioxide and ferricyanide have been used as oxidising agents. All the agents oxidise both adrenaline and noradrenaline at a pH above 5 but only adrenaline is oxidised below pH 4, a property used for their differential estimation. The pink coloured quinones produced by this oxidation can be transformed to highly fluorescent derivatives by the addition of alkali and the exclusion of oxygen. Estimation of individual catecholamines is by dual oxidations at pH 3 and pH 6. Alternatively, adrenaline and noradrenaline may be measured separately using differential spectrofluorimetry, reading at different activating and emission wavelengths (Vendsalu, 1960)¹⁵.

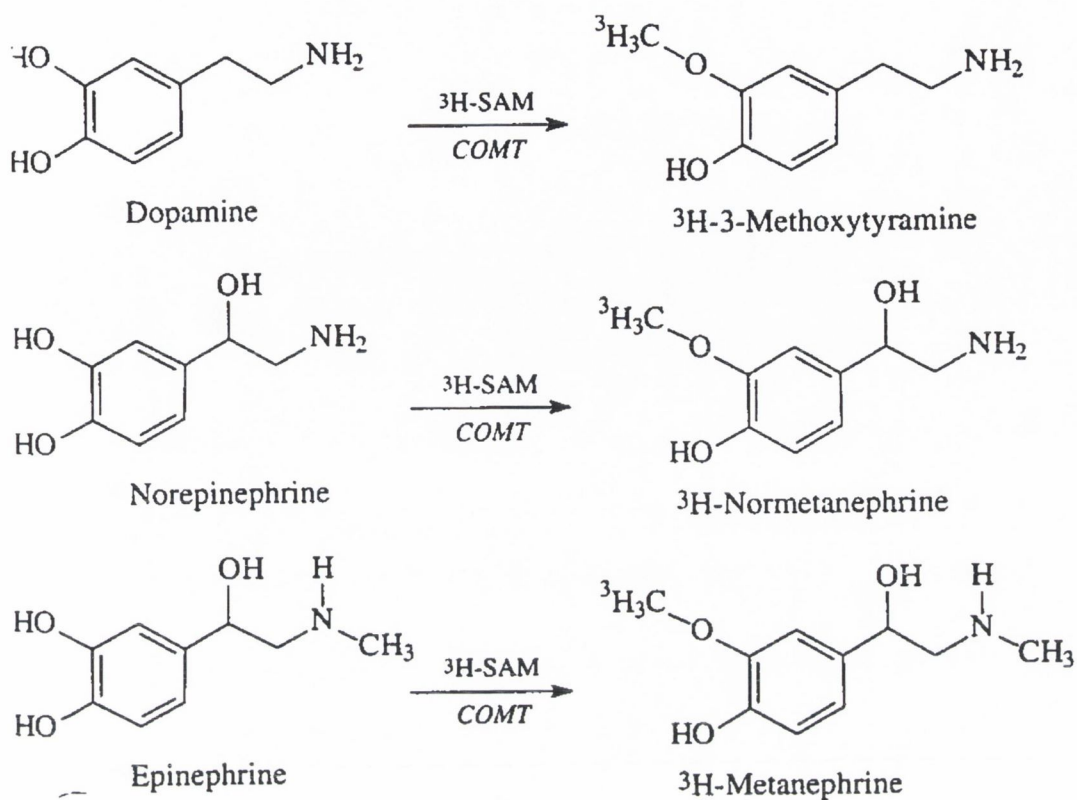


In the ethylenediamine (EDA) method, noradrenaline and adrenaline are first oxidized to adrenochromes and there is condensation of the two *o*-quinone groups of adrenochrome with ethylenediamine in alkaline solution in which adrenaline readily undergoes auto-oxidation¹⁶. The fluorescent condensation products have different emission spectra thus allowing both hormones to be measured simultaneously. Dopamine and some of its derivatives, which may be present in urine, also give a strongly fluorescent product in this reaction. Sample pretreatment with alumina, cation-exchange resins or both is necessary. Neither method is sensitive or specific for plasma adrenaline or noradrenaline. Common drugs such as ampicillin, methyl dopa, promethazine, sulphonamides and vitamin B complexes and coffee, tea and cocoa may interfere with fluorometric methods.

Reference interval with the EDA method is 140 – 300 pg/ml for adrenaline and 360 – 800 pg/ml for noradrenaline¹⁷.

(ii) Radioenzymatic

The original radioenzymatic assay was reported by Engelman et al¹⁸. The principal of this technique is based upon the use of catechol-O-methyltransferase (COMT) to transfer a radioactive methyl group from the tritiated reagent S-adenosyl-methionine to catecholamines present in the sample, forming their corresponding labeled methylated derivatives. Peuler's method is now commercially available¹⁹.



After the formation of O-methylated derivatives, the products are extracted into organic solvents and separated using thin layer chromatography on the basis of polarity of their side chains. The individual compounds are removed from the gel plate by scraping and direct measurement is made of the radioactivity of the tritium labelled 3-methoxytyramine fraction and provides quantitation for dopamine. The separated labelled normetanephrine and metanephrine are further converted by periodate oxidation to tritium-labelled vanillin which is directly proportional to the amount of noradrenaline or adrenaline. Quantitation of the individual catecholamines in the plasma is done by comparison with the radioactivity produced by corresponding internal standards in a duplicate system.

The radioenzymatic assay system is sensitive to 1pg (20 pg/mL of plasma) for noradrenaline and adrenaline, and to 6 pg (120pg/mL of plasma) for dopamine.

(iii) HPLC

Preliminary extraction and concentration of plasma is necessary to measure the very low plasma catecholamine levels in normal subjects. The principles are summarised in Teitz³. HPLC methods are generally more specific than radioenzymatic assays, and the values obtained are considerably lower¹². Method modifications have focused primarily on extraction techniques and chromatographic conditions.

A new automated method involving a second generation automated sequential trace enrichment of dialysates (ASTED) has been developed. It uses two rheodyne valves and two dilutors. The plasma sample is dialysed to remove protein and other macromolecules. The catecholamines are then trace-enriched onto the analytical column. Separation is by reversed-phase ion-pair chromatography with coulometric detection. The trace enrichment cartridge and dialyser are regenerated for subsequent injections. The procedure gave good agreement with manual alumina extraction methods²⁰.

(iv) **Gas Chromatography**

Gas chromatography with flame ionisation, electron capture or mass fragmentographic detection^{21,22}. As catecholamines are polar, non-volatile, unstable and relatively insoluble in most solvents, they do not lend themselves well to gas chromatography without prior derivatisation. Plasma deproteinisation and alumina adsorption are generally used for sample pretreatment followed by derivatization. The whole spectrum of derivatives used for catecholamines and metabolites include TMS ether, isothiocyanate, cyclic boronate, acetates, propionylate, butyrate and condensation products of TMS ethers. Two effective means of isolating catecholamines and metabolites from plasma and urine are cation exchange resins and ion-paired extraction. Although the use of catecholamine trifluoroacetyl, pentafluoropropionyl, heptafluorobutyl or perfluorobenzoyl derivatives on electrochemical detectors provides the sensitivity needed for plasma, the high noise level due to derivatisation reagents and contaminants from the sample matrix limit the general use of such these methods. Gas chromatography- mass spectrometry has the sensitivity and specificity of a reference method²³.

Reference Values

A literature review of adult reference intervals for noradrenaline and adrenaline in plasma from healthy normotensive subjects in the supine position is shown in a table taken from Rosano et al²⁴.

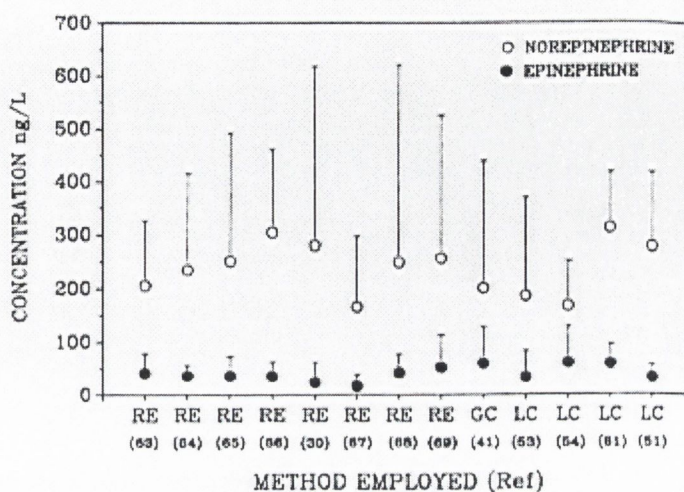


Fig. 2. Comparison of reference intervals for plasma norepinephrine and epinephrine in supine normotensive subjects
Data from a literature survey of studies with radioenzymatic (RE), gas-chromatographic (GC), and high-performance liquid chromatographic (LC) methods are expressed as mean plus 2 SD (bars). The reference source is cited in parentheses

Determination of Urinary Catecholamines

Diurnal variation has been shown in noradrenaline and adrenaline but not in dopamine excretion with higher levels while awake²⁵. Reference ranges for urinary catecholamines, HMMA and HVA in children have been published and reviewed^{26,27}. Adult reference ranges are treated separately in this document.

Total urinary catecholamines can be measured by first hydrolyzing the sample prior to assay. However as free catecholamines reflect the tumour load in pheochromocytoma and are least affected by dietary changes, free catecholamines are usually measured in diagnosis. All urinary methods require the initial removal of interfering substances. Ion-exchange chromatography and adsorption chromatography are most commonly used. Alumina pretreatment using a batch extraction technique at pH 8.6 and then elution with hydrochloric acid is often used. Boric acid affinity gels provide for selective adsorption of catecholamines. Commercial available columns are available using immobilized boric acid (Affigel 601) or phenylboric acid (Amicon 30).

Fluorescence methods

The trihydroxyindole method is adequate for urine testing but drugs such as α -methyldopa, quinidine, propranolol, labetalol, isoprenaline and tricyclic antidepressants which also show fluorescent properties may interfere to cause false high results²⁸. Other substances may quench fluorescence causing false negatives. Modifying the original methods to include iodine as an oxidizing agent allows for the additional measurement of dopamine²⁹.

HPLC

HPLC with fluorescent detection is more sensitive after pre-treatment with isolation by cation exchange and concentration on alumina. Pre- and post- column derivatizations may be used to improve method sensitivity²⁵.

Co-analysis of catecholamines, metanephrine and normetanephrine is possible by combining sequential chromatographic separation, electrochemical oxidation and derivatization with 1,2-diphenylethyleneamine to fluorescence³⁰.

Ion-pair adaptations of reversed phase chromatography with electrochemical detection are the most commonly used in clinical practice and forms the basis for our method described separately. Ion pairing with alkyl sulphonates or sulphates is usually used to enhance retention of cationic amine moieties on the lipophilic stationary phase. Chromatographic retentions may be altered by modification of ion-pairing agents, ionic strength or organic compounds³¹.

In the commonly used method of Moyer et al³², samples are initially purified by alumina and boric acid gel. Recoveries of the catecholamines and the 3,4-dihydroxybenzylamine internal standard are about 60% except where urinary glucose is very high. Interferences may occur with α -methyldopa, paracetamol, labetalol and captopril.

It is possible to analyse the catecholamines and their *o*-methyated metabolites simultaneously with a single sample injection after a one-step sample preparation on Bio-Rex 70 cation-exchange resin³³. Chromatographic conditions have been published for the co-analysis of catecholamines, metanephrines, HMMA and 5-HIAA³⁴. A recent simple and flexible method has been developed for the quantification of catecholamines, metanephrines, 3-methoxytyramine, HMMA, HVA and 5-HIAA using a single mobile phase containing both sodium octane sulphonate and diethylamine as ion-pairing reagents and uses isocratic conditions. The between batch CVs in normal urine ranged from 6.5% for HMMA to 14.5% for adrenaline. Within batch, the CVs varied from 1.6% for HMMA to 6.5% for adrenaline³⁵.

Ion-exchange chromatography with electrochemical detection offers unique separation advantages over ion-pair reversed-phase chromatography when samples have been pre-treated by an immobilized phenylboric acid affinity column method³⁶.

The detection limits for catecholamines in picograms quantitated by different detectors were compared by Yui and Kawai³⁷.

Method	Noradrenaline	Adrenaline	Dopamine
Electrochemical	25	25	25
Ultra-violet	1400	1200	1250
Natural fluorescence	300	300	300
O-phthalaldehyde	75	-	130
Trihydroxyindole	1	1	800

The use of UV detection for the analysis of catecholamines is hampered by its insensitivity. Post-column derivatization with o-phthalaldehyde increases the sensitivity for noradrenaline and dopamine, but adrenaline is not measured because only primary amines are derivatized. The post-column derivatized THI method is the most sensitive described for HPLC measurements for noradrenaline and adrenaline but is insensitive with regard to dopamine. Electrochemical detection offers the advantage of measuring all the catecholamines with roughly equal and high sensitivity.

Capillary Electrophoresis³⁸

Capillary zone electrophoresis with photodiode array detection at 220 nm has been used to detect catecholamines and metabolites in human urine. The UV spectra of the catechols were detected at a concentration of 20 µm. The samples were first hydrolyzed by 0.5 M acid or base solutions. 3,4 dihydroxybenzylamine was used as an internal standard. The method was optimised for adrenaline, noradrenaline, normetanephrine, metanephrine, HMMA, dihydroxyphenylacetic acid (dopac), 5-HIAA, HVA and 3-methoxytyramic acid at pH 4.0 and 8.0 for their electrophoretic separation. Concentrations were at nmol level. The within-batch variation was <5%.

Chemiluminescence³⁹

This method assays total free catecholamines using sulphuric acid-derivitized beads for extracting and identifying catecholamine on the surface. Assay is by measuring the peroxide produced from catecholamine by chemiluminescence. The usual initial separation step is eliminated by identifying the two functional groups on the catecholamines using a derivitized bead. This is achieved by trapping the amino group on the catecholamine by ion binding with a sulphuric acid derivative immobilized on a bead and the diol of the catecholamine bound to the bead is converted to peroxide with imidazole under alkaline conditions. The peroxide produced was assayed by microflow injection-horseradish peroxidase-catalysed luminol chemiluminescence. The minimum detection limit of dopamine was 0.1 µmol/L and the within batch variation for dopamine (10.5 µmol/L) was 4.7%. Thus this method is applicable to the assay of total free catecholamine without the use of HPLC.

Immunoassay⁴⁰

ELISA kits are available for the measurement of urinary free catecholamines and metanephrines. A recent evaluation found no interference by common drugs. The intraassay coefficients of variation (CV) were 4.6 – 18% and the between batch CVs were 10-25%. The detection limits were 0.009 and 0.027 $\mu\text{mol/L}$ for free noradrenaline and adrenaline respectively and 0.119 and 0.346 $\mu\text{mol/L}$ for total metanephrine and normetanephrine respectively.

Determination of Metanephrine and Normetanephrine in Urine

Electrophoresis. ⁴¹

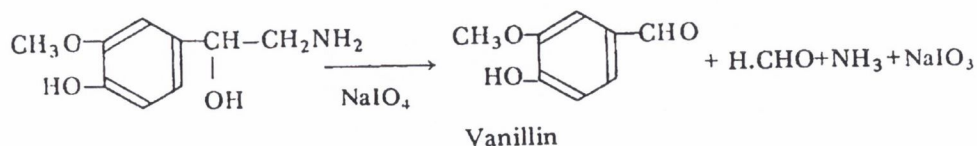
Paper⁴² and Thin Layer Chromatography⁴³

Spraying with dichloroquinone chlorimide or diazotised p-nitroaniline yielded chromogen products which can be measured spectrophotometrically. These techniques are no longer employed in clinical laboratories.

Colorimetric

Normetanephrine and metanephrine are excreted in the urine as free amines and as conjugates of glucuronide and sulphate. Total metanephrine excretion is not significantly influenced by diet⁴⁴. Most methods measure total metanephrines by initially hydrolysing the urine. An initial purification step, usually using weak cation-exchange resins is necessary. Differential solvent extraction methods can be used to remove potential interferences.

In 1960, Pisano⁴⁵ developed a method for determining normetanephrine and metanephrine in urine. After acid hydrolysis, the unconjugated metanephrines are adsorbed on to a cation – exchange resin (Amberlite CG 50), eluted with ammonium hydroxide and converted to vanillin by oxidation with periodate, the vanillin formed being read spectrophotometrically at 360nm. This method includes both normetanephrine and metanephrine together and is subject to negative interferences by phenoxy acids, radio-opaque x-ray dyes, propranolol and other drugs.



In 1986, Yates & Weinkove⁴⁶ published a modified method to make the analysis technically easier and to improve the precision and accuracy. This assay is unaffected by dietary vanillin, but suffers from interference by labetalol, atenolol, sotalol and phenolphthalein-containing compounds.

Fluorometric

These methods are more sensitive and specific than the Pisano colorimetric variance. Most fluorometric methods are based on chemical oxidation of metanephrines to their THI derivatives. Again, the urine samples are hydrolyzed and then partially purified by cation exchange chromatography before the oxidation reaction⁴⁷. Alternatively, the metanephrines may be quantitated using a dual-column extraction procedure where the total metanephrines are first partially purified on a strong cation exchange column and then fractionated on a weak ion-exchange resin before oxidation and fluorometric analysis. The stability of the THI derivatives is variable and the methods are labour intensive.

Radioenzymatic

These methods are available but are technically complicated⁴⁸. They do not quantify metanephrine and commonly used antihypertensives do not interfere in the assay.

Radioimmunoassay

Radioimmunoassays have also been developed for metanephrine and normetanephrine⁴⁹. ¹²⁵I-labelled octopamine and synephrine are used as heterogeneous tracers as normetanephrine and metanephrine could not be iodinated⁵⁰.

Enzyme Immunoassay

An ELISA assay using a microtitre plate has recently been described for normetanephrine and metanephrine⁵¹. For metanephrine, published within batch CVs vary from 6.42% to 9.47% and between batch vary from 10.1% to 14.3%. Similar values for normetanephrine are 6.76% to 9.22% and 9.19% to 12.5%. The correlation with GCMS methods were good – Correlation coefficients for patients and controls combined was 0.993 for metanephrine and 0.988 for normetanephrine. Passing-Bablok regression analysis comparing EMIT and GCMS methods, using patients and controls showed that there was no significant deviation from zero for the intercept and from 1.00 for the slope could be observed for the metanephrine and normetanephrine data.

A recent report shows no drug interference with new immunoassay methods⁴⁰.

Capillary Electrophoresis

Normetanephrine and metanephrine can be measured by capillary electrophoresis as described earlier in this chapter³⁸.

Gas Chromatography

These methods allow the estimation of total urine metanephrines⁵². Preliminary extractions are necessary to remove interfering catecholamines before the metanephrines are oxidised to vanillin. This is converted to its volatile silyl derivative and then detected with flame ionisation. Alternatively, trifluoroacetyl derivatives can be prepared and detected with mass spectrometry or electron capture. The necessary instrumentation is rarely available in hospital laboratories. However, the techniques are sensitive and specific.

HPLC

Column conditions and stationary phases vary. Common methods use reversed phase chromatography with ion-pairing reagents and silica-based cation-exchange chromatography. Fluorescence and ultraviolet detection systems may be used but most HPLC procedures use electrochemical detection. With sequential preliminary extraction, HPLC separation and electrochemical detection, the analyses are highly selective. A summary is provided in Tietz Textbook⁵³. The method currently used in the laboratory using the ASTED HPLC system is detailed.

Drug interferences with HPLC methods include viloxazine, paracetamol (false increase), labetalol and α -methyldopa.

Reference values

A table of published reference values for 24-hour urinary normetanephrine and metanephrine in normotensive people is shown (from Rosano et al). Values are higher in hypertensives^{54,55} and there is some diurnal variation which may affect the interpretation of results from short collection intervals⁵⁶.

Table 2. Reference Interval for Metanephrine Excretion in Urine

		Concn, $\mu\text{g}/24\text{ h}$				
Method	n	MN		NM		Total MN, mean (2 SD)
		Mean (2 SD)	Range	Mean (2 SD)	Range	
Spectrophotometry	30					600 (600)
	121					620 (560)
	18					500 (280)
Fluorometry	19	72 (50)	28–113	158 (152)	78–369	
	6	164 (52)	122–198	245 (144)	176–391	
	16	70 (18)	29–142	130 (32)	69–272	
GLC	18					670 (400)
	24	149 (112)	79–258	231 (134)	117–340	
HPLC	15	174 (158)	74–297	238 (160)	105–354	
RIA	22	154 (148)		217 (218)		
	30	172 (110)		96 (64)		261 (146)
Thin-layer chromatography	80	253 (180)		239 (202)		

Estimation of Urinary 4-Hydroxy-3-Methoxy-Mandelic Acid (HMMA/VMA)

A 24-hour urine collection is made under acidic conditions at 4° C to ensure stability. HMMA is not significantly conjugated and is therefore measured without a hydrolysis step.

4-Hydroxy-3-Methoxy-Mandelic Acid (HMMA) is often called vanillylmandelic acid (VMA). It is the major metabolite of catecholamines found in the urine and represents about 60% of the total excretion products derived from noradrenaline and adrenaline. Unlike metanephrines, HMMA is not significantly conjugated and is measured without a hydrolysis step. Most analytical procedures require an initial extraction step to remove interferences. This is usually an anion exchange.

Spectrophotometric

Sandler and Ruthven^{57,58} (1959, 1961) provided the first quantitative method for measuring HMMA in urine by adsorbing it on to Dowex 1 x 2 cation-exchange resin in the acetate form, followed by elution, whereas Pisano⁵⁹ et al (1962) used direct solvent extraction. In either case, periodate was used to oxidise the HMMA to vanillin which was assayed spectrophotometrically at 350 nm. With Pisano, HMMA and other phenolic acids are extracted into acidified urine with ethyl acetate and then re-extracted into aqueous potassium carbonate solution. The addition of sodium metaperiodate to the carbonate extract oxidizes HMMA to vanillin. Vanillin is then separated from contaminating phenolic acids by selective extraction into toluene and back extraction into carbonate before spectrophotometry. Dietary interference with this method is a major problem especially ingestion of chocolate, coffee, bananas, vanilla containing delicacies, aspirin and alpha methyl dopa. Dietary restrictions are unnecessary with chromatographic and electrophoretic techniques.

Paper chromatography / high voltage electrophoresis and thin layer chromatography

Two-dimensional chromatography was used for semi-quantitative work (Gitlow et al⁶⁰, 1960; Robinson et al⁶¹ 1959). Thin-layer chromatography (TLC) was also used⁶². Quantitation following TLC, derivatisation and scanning densitometry has been described⁶³. These methods have been superseded.

Gas Chromatography

GC with flame ionization or mass spectrometry detection has been used⁶⁴.

Column Chromatography

A column chromatography method for urinary HMMA has been described. Interfering substances are eliminated by passing the urine through a Dowex-1 x 2, 50 – 100 mesh

column and washing the column with distilled water. The HMMA is eluted from the resin using K_2CO_3 -containing 3.0 mol/L NaCl solution and water. Recoveries using standard addition were 92.1 to 100.4% with a mean of 97.3%. The within-batch and between-batch CVs were <5.5% and <6.95% respectively. No interfering effects were found with noradrenaline, adrenaline, aspirin or HVA up to quite high levels. The method correlated well with the commonly used Pisano's method⁶⁵.

HPLC

Most HPLC methods use reversed phase separation with various detection systems including ultra violet, fluorometric, electrochemical or post column reaction. Our method is described in detail in chapter 4.

Fluorescence polarization immunoassay

A rapid fluorescence polarization immunoassay for urinary HMMA was published in 1990 for use on the Abbott TDx fluorimeter⁶⁶. The minimum detectable concentration was 0.3 mg/L and the range of the standard curve was 0.3 to 200 mg/L. Antiserum from sheep was used.

Enzyme-linked immunoassay

An EIA method has been described⁶⁷ and used in mass screening in Austria⁶⁸. These monoclonal antibodies have been shown to have cross-reactivities against HVA and HMMA. The method requires back-up from HPLC with electrochemical detection or gas chromatography-mass spectrometry to exclude false positives⁶⁸. Method improvements have been described resulting in a cross reactivity of the HMMA assay of 0.8% with HVA and <0.4% with other structurally related catecholamine metabolites⁶⁹.

An enzyme-linked immunoadsorbent assay (ELISA) has been used for neuroblastoma screening and can be used with specimens dried on filter paper⁷⁰.

Capillary electrophoresis

This method has been developed for screening for metabolic disorders^{71,38}.

Pulse polarography⁷²

This uses a dropping mercury electrode where the electrode surface is continually renewed. This reduces electrode contamination by sample solutions such as proteins. The system used was the Mo(VI)-HMMA-bromate system. Polarographic measurements are carried out on a voltammetric analyser at 25° C with removal of dissolved oxygen. Results gave good agreement with HPLC methods.

Measurement of homovanillic acid (3-methoxy-4-hydroxyphenylacetic acid) (HVA) in urine

HVA is the main urinary metabolite of dopa and dopamine.

Spectrophotometric

Methods using the reaction of HVA with 1-nitroso-2-naphthol⁷³ were commonly used and an improved method using 1-nitroso-2-naphthol-4-sulphonic acid was published⁷⁴. It was more stable and was linear to HVA values of 100 µg per mg creatinine and had some interference from HMMA and metanephrines.

Chromatographic

Thin-layer chromatography methods are no longer used in clinical laboratories.

Gas chromatography⁷⁵ Using flame ionisation detection, a reported 'within batch' coefficient of variation (cv) for HMMA was 6% and for HVA was 5.5% while the 'between batch' cv was 7.2% and 6.4% respectively⁷⁶. However these methods for HVA and HMMA have been largely superseded in clinical practice by HPLC methods.

Gas chromatography with electron impact mass spectrometry has also been used for HVA as well as HMMA. Sample collection from soaked filter papers has been described⁷⁷.

HPLC

Preliminary clean-up is usually done with anion exchange chromatography. Separation is usually by reverse phase chromatography with ultraviolet or fluorescence spectrometry or amperometry. HPLC with electrochemical detection show little interference and our method (details shown) gives simultaneous separation of HMMA and 5-hydroxyindoleacetic acid.

ELISA (enzyme-linked immunosorbent assay)

An ELISA method using a monoclonal antibody to a conjugate of HVA-ovalbumin has been described. It cross reacts with HMMA and normetanephrine at 0.18% and <0.1% respectively. The assay coefficients of variation were <10% within the working range of 0.5 – 40 mg/L. This method is suitable for use in mass screening of infants for neuroblastoma⁷⁸.

Capillary electrophoresis

This method with photodiode array detection at 220 nm has been recently described³⁸.

Methods for measuring urinary 5-hydroxyindoleacetic acid (5-HIAA)

Methods for the estimation of urinary 5-HIAA have been well reviewed by Deacon⁷⁹.

Spectrophotometric method using the reaction of 5-HIAA with 1-nitroso-2-naphthol and nitrous acid reagent. Excess yellow reagent is removed with ethyl acetate. This test can be made approximately quantitative and may be used as a screening test for carcinoid syndromes. The reaction is not specific for 5-HIAA and also occurs with any 5-hydroxyindole, many guaiacol derivatives and para-substituted phenols and guaiacol derivatives.

Using a more specific method for 5-HIAA, keto-acids in the urine, which cause negative interference, are first removed with 2,4-dinitrophenylhydrazine. Indoleacetic acid is extracted into chloroform. Then the solution is saturated with sodium chloride, 5-HIAA is extracted into diethylether and buffered to pH 7.0 with phosphate buffer and read colorimetrically⁸⁰.

Ehrlich's aldehyde reagent (4-dimethylaminobenzaldehyde)

5-HIAA forms a blue colour when boiled with this reagent. This may be used as a qualitative or quantitative test. For quantitation, the 5-HIAA is extracted into diethylether and dried down. The residue is dissolved into HCl and reacted with Ehrlich's reagent. The reaction is poorly understood but appears to be given by compounds containing a pyrrole ring with a free α hydrogen.

Reaction with ferric chloride

The hydroxyl group on 5-HIAA reacts with ferric chloride to form a chromophore, the absorbance of which can be measured at 510 nm. This reagent is widely used for the detection of phenolic derivatives and acetoacetic acid but the reaction is fairly non-specific. Specificity can be improved by using acid conditions to carry out the reaction. Salicylate, for example, then no longer interferes⁸¹.

Reaction with 3-methyl-2-benzothiazolone MBTH.

5-HIAA is oxidatively coupled to this reagent using sodium periodate to form a strongly absorbing complex which is measured at 510nm. Tyrosine and salicylate are reported not to interfere with this method⁸².

To avoid falsely elevated values, patients must avoid drugs such as phenobarbitone, reserpine and chlorpromazine and such common foods as bananas, avocados, plums, walnuts, pineapple and tomatoes.

Fluorometric

Fluorometric – 5-HIAA is strongly fluorometric over the pH range 2 to 11. It is maximally excited at 295 nm and fluoresces at 330nm. These methods are sensitive. There is a problem with many fluorescing compounds in urine. Various sample preparations and treatments have been used to improve specificity and reduce interferences⁷⁹.

Radioimmunoassay

A method was described in 1981⁸³ but it has been superseded in clinical laboratory practice.

Fluoroimmunoassay

An FPIA method was published in 1989⁸⁴. 5-HIAA competes with fluorescein labelled 5-HIAA for antibody binding sites. Binding of the labelled 5-HIAA increases the rotational relaxation time relative to the fluorescence decay time, resulting in an increase in the degree of polarization of the emitted light. The degree of polarization is inversely proportional to the concentration of 5-HIAA in the sample. The reagents are stable and infrequent calibrations are required. There was no demonstrable drug interference. The method is commercially available for the Abbott TDx analyser and is reported to compare favourably with HPLC⁸⁵.

Paper/Thin Layer Chromatography

Two dimensional paper chromatography can be used to separate 5-HIAA from the other urinary indoles. An advantage of these methods is that other indoles can be evaluated simultaneously. Ehrlich's reagent is usually used for identification and visualisation. The sensitivity can be improved by using 4-dimethylaminocinnamaldehyde instead of 4-dimethylaminobenzaldehyde in Ehrlich's reagent⁸⁶.

Gas Chromatography with mass spectrometry⁸⁷

GC relies on the distribution of the 5-HIAA between a stationary liquid phase and the mobile gas phase. The liquid stationary phase can be coated onto either a packed or capillary column. Because 5-HIAA is non-volatile, it must be converted into a suitable volatile derivative for separation by GC. A preliminary purification step is essential. Reaction with pentafluoropropionic acid or N,O-bis(trimethylsilyl)trifluoroacetamide have been used for derivatisation. Both flame ionization and electron capture have been used for detection.

HPLC

Various HPLC methods are available with ultraviolet absorptiometry, fluorescence and electrochemical detection⁸⁸. The carboxyl group of 5-HIAA is dissociated at neutral pH, rendering the molecule too hydrophilic to be retained by a reversed-phase column and it elutes within the void volume. This problem is overcome by employing an acidic mobile

phase to suppress the ionization of the carboxyl group so that 5-HIAA can interact with the stationary phase and its passage through the column is retarded. Thus, the capacity factor increases as the pH of the mobile phase is reduced.

The HPLC method used in this laboratory gives simultaneous results for HMMA, HVA and 5-HIAA and is described in detail.

Capillary Electrophoresis

This has been described but there are problems with decomposition at the detection stage³⁸.

References

1. Lee MR. Dopamine and the kidney. Clin Sci 1982;62:439-48.
2. Brown RT, Polinsky RJ, Kopin IJ. Interconversion of *O*-methylated norepinephrine metabolites in humans. J Clin Lab Med 1987;110:119-24.
3. Biogenic amines in Tietz Text Book of Clinical Chemistry, 2nd Edition, Ed CA Burtis, ER Ashwood. 1994;WB Saunders, Philadelphia p1749.
4. Cannon WB, Rosenbleuth A. Studies on conditions of activity in endocrine glands. Am J Physiol 1933;104:557-74.
5. Booker WM, The isolated perfused guinea pig heart as a biological assay method for adrenaline and noradrenaline. Arch Int Pharmacodyn 1959;123:206-15.
6. Valette G, Nguyn-ba-Muoi. On the use of segments of rat aorta for the assay of adrenaline solutions. Ann Pharm Franc 1962;20:377-82
7. De La Lande IS, Harvey JA. A new and sensitive bioassay for catechol amines. J Pharm Pharmacol 1965;17:
8. Armitage AK, Vane JR. A sensitive method for the assay of catechol amines. Br J Pharmacol 1964;22:204-10.
9. Gaddum JH, Peart WS, Vogt M. The estimation of adrenaline and allied substances in the blood. J Physiol (Lond) 1949;108:476-81.
10. Pennefather JN, Rand MJ. Increase in the noradrenaline content of tissues after infusion of noradrenaline, adrenaline and L-dopa. J Physiol (Lond) 1960;154:277-87.
11. Vane JR. The estimation of catecholamines by biological assay. Pharmacol Rev 1966;18:317-24.

12. Von Euler US, Hamberg U. *Science* 1949;110:561.
13. Crout JR. *Standard methods in clinical chemistry*. Vol 3 Seligson D, ed 1961 p62, New York, Academic Press.
14. Griffiths JC, Leung FY, McDonald TJ. Fluorimetric determination of plasma catecholamines: normal human epinephrine and norepinephrine levels. *Clin Chim Acta* 1970;30:395-405.
15. Vendsalu A. *Acta Physiol Scand*. 1960;49:Suppl 173.
16. Manger WM, Steinsland OS, Nahas GG, Wakim KG, Dufton S. Comparison of improved fluorometric methods used to quantitate plasma catecholamines. *Clin Chem* 1969;15:1101-23.
17. Weil-Malherbe H. The chemical estimation of catecholamines and their metabolites in body fluids and tissue extracts. *Methods Biochem Anal* 1971;Suppl:119-52.
18. Engelman K, Portnoy B, Lovenberg W. A sensitive and specific double-isotope derivative method for the determination of catecholamines in biological specimens. *Am J Med Sci* 1968;255:259-68.
19. Peuler JD, Johnson GA. Simultaneous single isotope radioenzymatic assay of plasma norepinephrine, epinephrine and dopamine. *Life Sci* 1977;21:625-36.
20. Dutton J, Hodgkinson AJ, Hutchinson G, Roberts NB. Evaluation of a new method for the analysis of free catecholamines in plasma using automated sample trace enrichment with dialysis and HPLC. *Clin Chem* 1999;45:394-9.
21. Eisenhower G. Analytical differences between the determination of plasma catecholamines by liquid chromatography with electrochemical detection and by radioenzymatic assay. *J Chromatog* 1986;377:328-33.
22. Jacob K, Vogt W, Knedel M, Schwertfeger G. Quantitation of adrenaline and noradrenaline from human plasma by combined gas chromatography high retention mass fragmentography. *J Chromatog* 1978;146:221-6.
23. Lovelady HG, Foster LL. Quantitative determination of epinephrine and norepinephrine in the picogram range by flame ionization gas-liquid chromatography. *J Chromatog* 1975;108:43-52.
24. Chakraborty J. *Gas Chromatography*. In: *A Guide to Gas-liquid and high performance liquid chromatography*. 1987: ACB, London,18-65
25. RosanoTG, Swift TA, Hayes LW. Advances in catecholamine and metabolite measurement for diagnosis of pheochromocytoma. *Clin Chem* 1991;37:1854-67.

26. FitzGibbon M, FitzGerald RJ, Tormey WP, O'Meara A, Kenny D. Reference values for urinary HMMA, HVA, noradrenaline, adrenaline and dopamine excretion in children using random urine samples and HPLC with electrochemical detection. *Ann Clin Biochem* 1992;29:400-4.
27. FitzGibbon MC, Tormey WP. Paediatric reference ranges for urinary catecholamine/metabolites and their relevance in neuroblastoma diagnosis. *Ann Clin Biochem* 1994;31:1-11.
28. Kahane Z, Vetergaard P. An improved method for measurement of free epinephrine and norepinephrine with a phosphate-meta-phosphate buffer in the trihydroxyindole procedure. *J Lab Clin Med* 1965;65:848-58.
29. Oka K, Sekiya M, Osada H, Fujita K, Kato T, Nagatsu T. Simultaneous fluorometry of urinary dopamine, norepinephrine, and epinephrine compared with liquid chromatography with electrochemical detection. *Clin Chem* 1982;28:646-9.
30. Nohta H, Yamaguchi E, Ohkura Y, Watanabe H. Simultaneous high-performance liquid chromatographic determination of catecholamine-related compounds by post-column derivatization involving coulometric oxidation followed by fluorescence reaction. *J Chromatogr* 1989;467:237-47.
31. Kontur P, Dawson R, Monjan A. Manipulation of mobile phase parameters for HPLC separation of endogenous monoamines in rat brain tissue. [Review] *J Neurosci Methods* 1984;11:5-18.
32. Moyer TP, Jiang N-S, Tyce GM, Sheps SG. Analysis for urinary catecholamines by liquid chromatography with amperometric detection: methodology and clinical interpretation of results. *Clin Chem* 1979;25:256-63.
33. Chan YP, Siu TS. Simultaneous quantitation of catecholamines and *O*-methylated metabolites in urine by isocratic ion-pairing high-performance liquid chromatography with amperometric detection. *J Chromatogr* 1988;459:251-60.
34. Parker NC, Levtzow CB, Wright PW, Woodward LL, Chapman JF. Uniform chromatographic conditions for quantifying urinary catecholamines, metanephrines, vanillylmandelic acid, 5-hydroxyindoleacetic acid, by liquid chromatography with electrochemical detection. *Clin Chem* 1986;32:1473-6.
35. Radjaipour M, Raster H, Liebich HM. Quantification of urinary catecholamines, their abundant metabolites, and 5-hydroxyindole acetic acid by high performance liquid chromatography and electrochemical detection, using a single mobile phase and uniform isocratic conditions. *Eur J Clin Chem Clin Biochem* 1994;32:609-13.

36. Wu AHB, Gornet TG. Preparation of urine samples for liquid-chromatographic determination of catecholamines: bonded-phase phenylboronic acid, cation-exchange resin, and alumina adsorbents compared. *Clin Chem* 1985;31:298-302.
37. Yui Y, Kawai C. Comparison of the sensitivity of various post-column methods for catecholamine analysis by high-performance liquid chromatography. *J Chromatog* 1981;206:586-8.
38. Siren H, Karjalainen U. Study of catecholamines in patient urine samples by capillary electrophoresis. *J Chromatog A* 1998;53:527-33.
39. Nozaki O, Kawamoto H, Moriyama H. Total free catecholamines assay by identification of its two functional groups and micro-flow injection chemiluminescence. *Luminescence* 1999;14:369-74.
40. Wassell J, Reed P, Kane J, Weinkove C. Freedom from drug interference in new immunoassays for urinary catecholamines and metanephrines. *Clin Chem* 1999;45:2216-23.
41. Yoshinaga K, Itoh C, Ishida N, Sato T, Wada Y. Quantitative determination of metadrenaline and normetadrenaline in normal human urine. *Nature* 1961;191:599-600.
42. Coward RF, Smith P. Recovery of total metanephrines from urine and their estimation by paper chromatography. *Clin Chim Acta* 1966;14:672-8.
43. Gutteridge JMC. Thin-layer chromatographic techniques for the investigation of abnormal urinary catecholamine metabolite patterns. *Clin Chim Acta* 1968;21:211-6.
44. Moleman P. Effect of diet on urinary excretion of unconjugated catecholamines and some of their metabolites in healthy control subjects and depressed patients. *Clin Chim Acta* 1990;189:19-24.
45. Pisano JJ. A simple analysis for normetanephrine and metanephrine in urine. *Clin Chim Acta* 1960;5:406-14.
46. Yates P, Weinkove C. Simplification of a urinary metadrenaline assay. *Ann Clin Biochem* 1986;23:487-9.
47. Kahane Z, Vestergaard P. Column chromatographic separation and fluorometric estimation of metanephrine and normetanephrine in urine. *Clin Chim Acta* 1969;25:453-8.
48. Kobayashi K, Foti A, DeQuattro V, Kolloch R, Miano L. A radioenzymatic assay for free and conjugated normetanephrine and octopamine excretion in man. *Clin Chim Acta* 1980;107:163-73.

49. Iinuma K, Ikeda I, Ogihara T, Hashizume K, Kurata K, Kumahara Y. Radioimmunoassay of metanephrine and normetanephrine for diagnosis of pheochromocytoma. *Clin Chem* 1986;32:1879-83.
50. Knoll E, Wisser H. Problems in the development of radioimmunoassay. *J Clin Chem Biochem* 1984;22:741-49.
51. Wolthers BG, Kema IP, Volmer M, Wesemann R, Westermann J, Manz B. Evaluation of urinary metanephrine and normetadrenaline enzyme immunoassay (ELISA) kits by comparison with isotope dilution mass spectrometry. *Clin Chem* 1997;43:114-20.
52. Calseyde JF, van de Scholtis RJ, Schmidt NA, Leyten CJJA. Gas chromatography in the estimation of urinary metanephrines and VMA. *Clin Chim Acta* 1971;32:361-6.
53. Determination of urinary metanephrine and normetanephrine In: Tietz Textbook of Clinical Chemistry, 2 ed. CA Burtis , ER Ashwood eds. 1994 WB Saunders, Philadelphia p1757-9.
54. Kobayashi K, Kolloch R, DeQuattro V, Miano L. Increased plasma and urinary normetanephrine in young patients with primary hypertension. *Clin Sci* 1979;57:173-6.
55. Foti A, Adachi M, DeQuattro V. The relationships of free to conjugated normetanephrine in plasma and spinal fluid of hypertensive patients. *J Clin Endocrinol Metab* 1982;55:81-5.
56. Taniguchi K, Kakimoto Y, Armstrong MD. Quantitative determination of metanephrine and normetanephrine in urine. *J Lab Clin Med* 1964;64:469-84.
57. Sandler M, Ruthven CRJ. Colorimetric estimation of 3-methoxy-4-hydroxymandelic acid in urine. *Lancet* 1959;113, 1034.
58. Sandler M, Ruthven CRJ. *Biochem J*; 1961;80:78.
59. Pisano JJ, Crout JR, Abraham D. Determination of 3-methoxy-4-hydroxymandelic acid in urine. *Clin Chim Acta* 1962;7:285-91.
60. Gitlow SE, Mendlowitz M, Khassis S, Cohen G, Sha J. The diagnosis of pheochromocytoma by determination of urinary 3-methoxy-4-hydroxymandelic acid. *J Clin Invest* 1960;39:221-5.
61. Robinson R, Ratcliffe J, Smith P. A screening test for phaeochromocytoma. *J Clin Pathol* 1959;12:541-43.

62. Preston JA. 3-methoxy-4-hydroxymandelic acid (VMA) by microfiber chromatography. Clin Chem 1967;13:19-25.
63. Agbaba D, Stojanov M, Rajacic S, Zivanov-Stakic D, Majkic-Singh N. Densitometric determination of urinary 4-hydroxy-3-methoxymandelic acid (vanillylmandelic acid). Clin Chem 1993;39:2500-3.
64. Tuchman M, Crippin PJ, Krivit W. Capillary gas chromatographic determination of urinary homovanillic acid and vanillylmandelic acid. Clin Chem 1983;29:828-31.
65. Thuvasethakul P, Manakul S. Urinary vanilmandelic acid determination using column chromatography. J Med Assoc Thai 1992;75 suppl 1:157-67.
66. Mellor GW, Gallacher G. Fluorescence polarization immunoassay of urinary vanillylmandelic acid. Clin Chem 1990;36:110-112.
67. Yokomori K, Hori T, Tsuchida Y, Kuroda M, Yoshioka M. A new urinary mass screening system for neuroblastoma in infancy by use of monoclonal antibodies against VMA and HVA. J Pediatr Surg 1989;24:391-4.
68. Kerbl R, Urban CE, Ambros PF, Lackner H, Ladenstein R, Spuller E, Mutz I, Ambros I, Amann G, Gadner H, Parker L. Screening for neuroblastoma in late infancy by use of EIA (enzyme-linked immunoassay) method: 115,000 screened infants in Austria. Eur J Cancer 1996;32A:2298-305.
69. Taran F, Bernard H, Valleix A, Creminon C, Grassi J, Olichon D, Deverre JR, Pradelles P. Competitive enzyme immunoassay for urinary vanillylmandelic acid. Clin Chim Acta 1997;264:177-92.
70. Ater JL, Gardner KL, Foxhall LE, Therrell BL jr, Bleyer WA. Neuroblastoma screening in the United States: results of the Texas Outreach Program for neuroblastoma screening. Cancer 1998;82:1593-602.
71. Presto Elgstoen KB, Jellum E. Capillary electrophoresis for diagnosis of metabolic disease. Electrophoresis. 1997;18:1857-60.
72. Hasebe K, Kakizaki T, Yoshida H. Determination of vanillylmandelic acid in urine by use of catalytic maximum wave in differential pulse polarography. Anal Chem 1987;59:373-6.
73. Goldenberg H. Specificity of the nitrosonaphthol reaction. Detection of metanephrine and other guaiacol derivatives. Clin Chem 1967;13:698.
74. Knight JA, Haymond RE. Improved colorimetry of urinary 3-methoxy-4-hydroxyphenylacetic acid (Homovanillic Acid). Clin Chem 1977;23:2007-10.

75. Brewster MA, Berry DH, Moriarty M. Urinary 3-methoxy-4-hydroxyphenylacetic acid (homovanillic) and 3-methoxy-4-hydroxymandelic (vanillylmandelic) acids: Gas chromatographic methods and experience with 13 cases of neuroblastoma. *Clin Chem* 1977;23:2247-9.
76. Taylor GF, Duong T, Carter NG. An improved gas chromatographic determination of urinary catecholamine metabolites. *Ann Clin Biochem* 1983;20:289-93.
77. Fauler G, Leis HJ, Huber E, Schellauf C, Kerbl R, Urban C, Gleispach H. determination of homovanillic acid and vanillylmandelic acid in neuroblastoma screening by stable isotope dilution GC-MS. *J Mass Spectrom* 1997;32:507-14.
78. Shi RZ, Ho Y-P, Yeung JHK, Or PMY, To KKW, Lau MWM, Arumanayagam M. Development of an enzyme-linked immunosorbent assay with monoclonal antibody for quantification of homovanillic in human urine samples. *Clin Chem* 1998;44:1674-9.
79. Deacon AC. The measurement of 5-hydroxyindole acetic acid in urine. *Ann Clin Biochem* 1994;31:215-32.
80. Udenfriend S, Titus E, Weissbach H. The identification of 5-hydroxy-3-indoleacetic acid in normal urine and a method for its assay. *J Biol Chem* 1955;216:499-505.
81. Yamaguchi Y, Hayashi C. Simple determination of high urinary excretion of 5-hydroxyindoleacetic acid with ferric chloride. *Clin Chem* 1978;24:149-50.
82. Mukerjee H, Pincus MR. A colorimetric determination of 5-hydroxyindoleacetic acid in urine. *Clin Chim Acta* 1992;209:105-6.
83. Puizillot JJ, Delaage MA. Radioimmunoassay of 5-hydroxyindoleacetic acid using an iodinated derivative. *J Pharmacol Exp Ther* 1981;217:791-7.
84. Brashear J, Zeitvogel C, Jackson J, Flentge C, Janulis L, Cantrell L, Schmidt B, Adamczyk M, Betebenner D, Vaughan K. Fluorescence polarization immunoassay of urinary 5-hydroxy-3-indoleacetic acid. *Clin Chem* 1989;35:355-9.
85. Kerswill W. Estimation of 5-hydroxy-3-indoleacetic acid by fluorescence polarization immunoassay *Clin Biochem Rev* 1991;12:65.
86. Berry K, Krupanidhi I. Estimation of urinary 5-hydroxyindoleacetic acid by paper chromatography. *Clin Chem* 1965;11:465-70.
87. Hoskins JA, Politt RJ. Quantitative aspects of urinary indole-3-acetic acid and 5-hydroxyindole-3-acetic acid excretion. *J Chromatogr* 1981;109:436-8.

88. Quigley ME, Lawson GJ. Urinary 5-hydroxyindoleacetic acid by HPLC with electrochemical detection. Clin Chem 1989;35:507

Laboratory methods used during the present studies

High performance liquid chromatography (HPLC) is the basis of the methods used. The principles are described¹. Separation is based on the distribution of the solutes between a liquid mobile phase and a stationary phase. Column efficiency is inversely related to the particle size of the column packing and in an efficient column relatively high pressure has to be used to pump the liquid mobile phase through the column. Basic systems consist of a solvent reservoir containing the mobile phase. This may be a glass flask into which feed lines to the pump are inserted. In-line filters are placed on the inlet of the feed lines to remove particles from solvents. Gas lines are often inserted into the reservoir to purge the solvents continuously with an inert gas like helium. This degassing is necessary to avoid an unstable baseline because bubbles of dissolved gas may evolve as the mobile phase passes from the high pressure to ambient pressure on the postcolumn side of the system. This is the system which has been used in this laboratory. Helium sparging requires a constant gentle stream of helium bubbled through the mobile phase in the reservoir during active separation. The relatively insoluble helium extracts dissolved gases. A drying agent is sometimes placed in the helium line between the helium tank and the solvent reservoir to remove any water in the gas.

A pump is needed to pump the liquid mobile phase through the packed column. The pump is used in either the isocratic or gradient mode. In the isocratic mode (used here), the mobile phase remains unchanged during the whole separation run unlike the gradient method where the composition is changed over the separation. With isocratic systems, an on-line solvent recycler can be used to recycle mobile phase. The device is connected to the detector outlet and has a valve activated by the detector signal. During the run, when no solutes are being eluted, the recycler diverts the effluent back to the mobile-phase reservoir. When the detector signal exceeds a predetermined threshold, the recycler diverts the effluent to a waste container until the signal again falls below the threshold. This reduces consumption of the mobile phase, solvent costs and waste disposal.

Adequate mixing of the mobile phase must be done to prevent baseline instability.

An injector is used to put the sample aliquot into the column. A fixed loop injector is usually used in which the sample loop is rotated into the flowing stream of mobile phase and the sample is then swept along into the separation column. These injectors are precise, can be used at high pressures and can be run with computer programmes to alter the volumes when necessary. The Waters programmable Waters Intelligent Sample Processor (WISP) system is used here.

The HPLC column is also needed to separate the analytes. Most columns are made from 316 stainless steel. The internal diameters are from 2 to 5 mm and the lengths are from 100 to 250 mm. Zero dead volume column end-fittings with frits to retain the support particles are used to connect the column to the injector on the inlet end and to the detector on the outlet side. To preserve uniform flow and minimize peak broadening, the frit pore size should be about one quarter of the mean particle diameter. Small uniform mean particle diameters of 3, 5 and 10 μm provide efficient columns with acceptable back

pressures. Usually lower detection limits are achieved with columns having smaller internal diameters. The columns used here are 150 mm long with 4.6 mm internal diameter.

A guard or a pre-column is used to conserve the life of the analytical column. The pre-column is placed between the pump and the injector and is usually packed with silica to saturate the mobile phase with silica in silica based systems. A guard column is placed between the injector and the analytical column and is packed with a similar stationary phase to the analytical column. They collect particulate matter and any sample components with high affinity for the stationary phase such as proteins. The volume of mobile phase in the guard column should be minimised to reduce band widening by limiting to 4 to 6% of the total volume of the analytical column. A guard column as described below is used in our system. In a bonded-phase stationary phase, the stationary phase is chemically bonded to the surface of the silica particles through a silica ester or a silicone polymeric linkage. Bonded-phase packings are mechanically and chemically stable, have long lives and provide good chromatographic performance.

In normal phase HPLC, the functional groups of the stationary phase are polar relative to those of the mobile phase which usually consists of non-polar solvents. Silanol, amino and nitrile groups are commonly used examples in the normal phase. Reversed phase requires a non-polar stationary phase. The most commonly used is C18 type where octadecylsilane molecules are bonded to silica particles. This is the type of column used here and is called an ODS column. Reversed-phase column retention and selectivity can be altered by attaching different groups such as octyl, phenyl, cyanopropyl to the silica. Differences are also caused by the size of the silica support particles and the amount of bonded phase used. Polymeric packings are alternatives to silica. Chiral column packings have been used to separate enantiomers.

A detector and a computer to control the system and process the data is also necessary. Photometric, spectrophotometric, fluorometric and electrochemical detectors have been used and mass spectrometers are also used as detectors for liquid chromatographs. This laboratory uses electrochemical detection - details follow.

Sample Collection

Urine samples were collected in pre-acidified containers. A 6 N solution of hydrochloric acid was used for this purpose. The amount of acid added to the container varied, depending upon the volume of the collection. For MSU samples 0.5ml of 6N HCL was found to be adequate, 5ml being used for 12 hour or longer collections. Only samples of pH5 or less were suitable for analysis.

Over time, the methods have been adjusted as detectors and conditions have changed. The methods used from 1988 until 1995 are firstly described and the subsequent minor alterations to the procedures are delineated. Participation in the UK National External Quality Assurance scheme for these analytes has been a standard procedure. Typical reports are shown in the Appendix 1 at the end of this chapter.

Method for the simultaneous determination of urinary HMMA and HVA and 5-HIAA

HPLC with electrochemical detection is the basis of the method.

The use of ethyl acetate to remove interfering electroactive compounds in the urine is used in both this method and the free catecholamine method described subsequently. In this assay specificity is improved by employing a phosphate back extraction at pH 7.0. Calibration was by the method of standard addition, which minimises matrix effects and helps in peak identification. An internal standard is regularly used in chromatographic assays to serve two major functions. Firstly, it ensures correct injection onto the column, and secondly, it verifies the absolute recovery of the extraction method preceding column separation. Isovanillic acid (IsoVa) was selected for this assay as it is not present physiologically, pharmacologically, or in the diet. Also the retention time is suitably separated from other peaks.

Method

A stock combined standard contained 217.99 mg HMMA, 210.32 mg 5-HIAA and 200 mg HVA. These were made up to 1 litre with 0.16 mol/L boric acid, to a final concentration of 1.1mmol/L. These were stored in the refrigerator and prepared every 2 months. The stock internal standard consisted of 100 ml isovanillic acid in 100ml ethanol. The working internal standard is 0.1 ml of stock added to 10 ml of ethyl acetate. The working mobile phase contained 10.9 g potassium dihydrogen orthophosphate and 100mg EDTA. These were dissolved in 160 ml methanol and 10 ml of 2 molar orthophosphoric acid was added before making up to a final volume of 1 litre with water. This solution was filtered and degassed before introduction into the HPLC system. The phosphate buffer at pH 7, had a limited shelf life due to bacterial growth. To overcome this problem, 2 solutions were made up and stored, and combined to make the working solution. Solution (A) consisted of 9.073g of potassium dihydrogen orthophosphate in 1 litre of water, and solution (B) was made by dissolving 11.87g disodium hydrogen orthophosphate in 1 litre of water. For the working buffer, 41.3ml of solution A was made up to 100ml with solution B.

HPLC System

All solvents were filtered through a 0.22µm filter before use.

All columns were stored in 50/50 methanol/water. Each time the solvent was changed the recycle line was placed into the waste reservoir for at least half an hour. The reagent was recycled only after purging the lines. The mobile phase was recycled to cut down on running costs.

Column Regeneration

1. Water
2. 50/50 Methanol/water
3. Methanol
4. Methanol/Dichloromethane 50/50
5. Dichloromethane

Allowed approximately 20 minutes running time with each solvent and the procedure was then repeated in reverse through steps 5 – 1.

Sample Preparation

1.0ml of each urine for analysis was pipetted into each test tube and 100µl of stock combined standard was added alternate tubes i.e. “spiked urines”. A 200 µl aliquot of both spiked and unspiked urine was stabilised by the addition of 20µl of 1N hydrochloric acid. To this 1.0ml of working internal standard was added. The tubes were mixed for 15 seconds and then centrifuged. 200µl of the upper organic layer was then transferred to a fresh tube containing 1ml of the working phosphate buffer (pH 7.0). Each tube was then mixed for 15 seconds and centrifuged again. The ethyl acetate layer was removed by suction, and discarded, and 20µl of 1N hydrochloric acid was then added to the remaining extract and mixed immediately. 25µl of each prepared sample was injected onto the column under the following chromatographic conditions:-

Column	:	Bio-Rad 5u C
Flow Rate	:	Generally 1.25ml/min
Electrochemical detector	:	Potential + 0.76 volts
		Filter 0.5 secs
		Range 0 – 250nA
		Offset 0 (set)

To enable the assay of small samples the sample volume was reduced from 1ml spiked with 100µl of standard to 0.5ml spiked with 50µl of standard.

Calculations

Calibration is by the method of standard addition of peak height ratios.

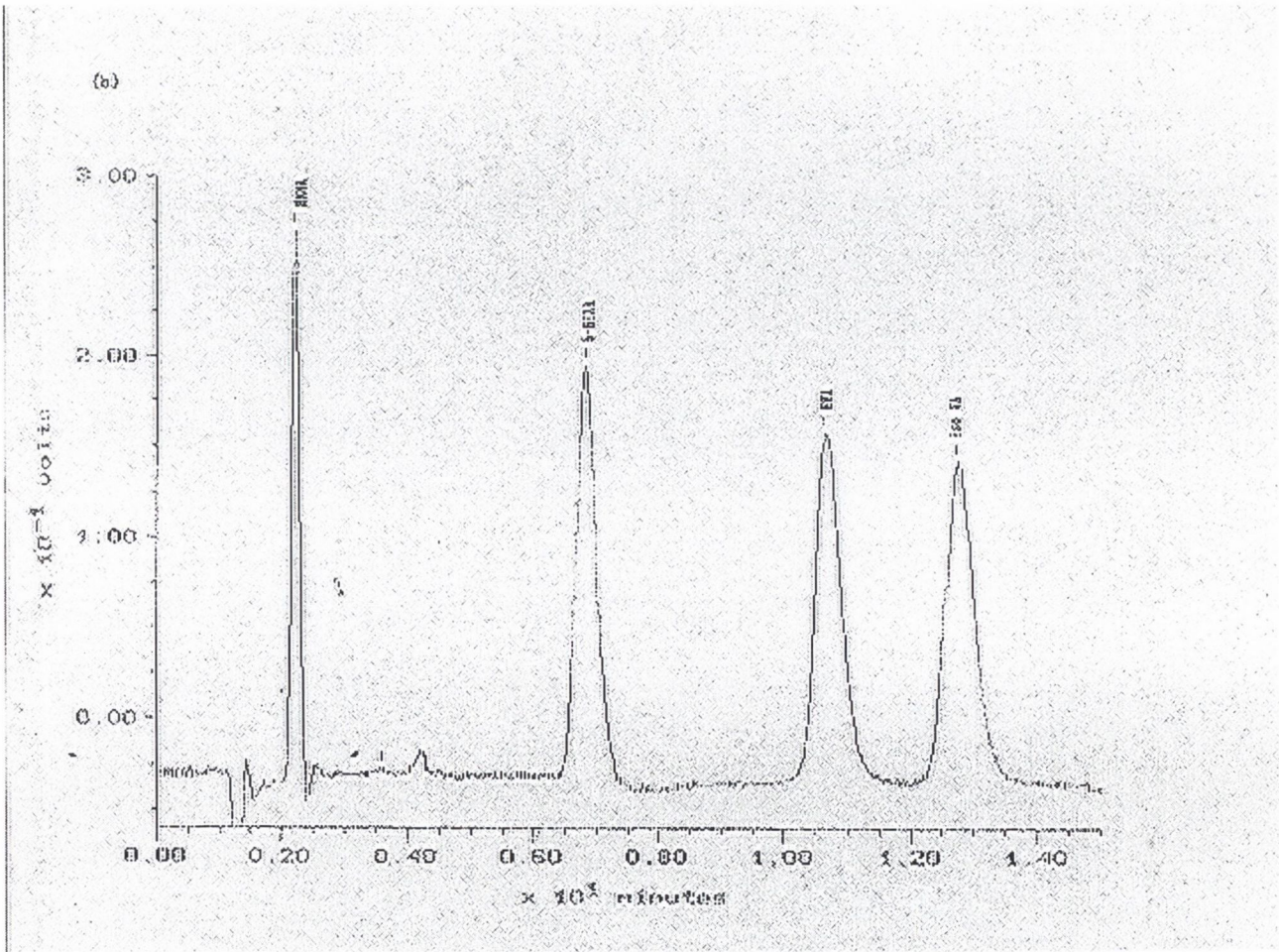
Potential Interferences

Paracetamol does not interfere with the assay, but it shows a peak at about twice the retention time of HMMA and a further peak, thought to be a metabolite, just after 5-HIAA. Patients taking atenolol may show a peak at the retention time of 5-HIAA when detected at +0.80volts. Davidson found that pure atenolol did not produce such a peak,

but a minor metabolite 4-hydroxyphenyl-acetic acid did². The latter interference was eliminated by reducing the voltage to +0.67.

Retention Times of metabolites HMMA, HVA, 5-HIAA and Iso Vanillic Acid

<i>Metabolite</i>	<i>Retention Time / Minutes</i>
HMMA	between 2 and 3 minutes
5-HIAA	between 6 and 7 minutes
HVA	approximately 11 minutes
Isovanillic acid	from 13 minutes onwards

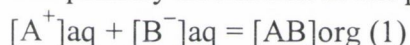


Method for the determination of urine free catecholamines by HPLC and ECD

Urine samples were collected as described earlier. Electrochemical detection with ion-pairing adaptation of reversed-phase chromatography is the basis of the method used in this laboratory. Reversed phase chromatography is so-called because the stationary phase (ie the column particles) is coated with carbon chains usually 18 carbon moieties in length (C18 columns). These columns provide efficient separation of compounds with varying lipophilicity, and absorption and the system is very versatile. Other factors also contribute to the separation. Reversed phase chromatograms often display narrow distinct peaks. The retention of the catecholamines may be altered by varying the pH of the mobile phase which affects the ionisation of the catecholamines, by adding a counter-ion to retain the catecholamines by ion pair extraction, and by adding an organic modifier to the mobile phase which reduces the retention on the reversed phase column.

Ion pair chromatography refers to ionic compounds that would normally be soluble only in aqueous phases, being rendered more soluble in organic solvents by the formation of hydrophobic "ion pairs" with an aqueous counter ion. The ion pair is thus able to partition between the organic and aqueous layers in much the same way as a neutral molecule.

The primary interaction in ion pair extraction may be written as follows:



where A^+ is the cationic protonated form of the base (ie the catecholamine) to be extracted, and B^- is the anion of an acid to be used as the pairing ion. (The roles of anion and cation can be reversed so that anionic species can be extracted by cationic pairing ions). As in any other reversible process, the extent of the forward reaction may be expressed quantitatively using the distribution coefficient K :

$$K = \frac{[AB]_{org}}{([A^+]_{aq}) ([B^-]_{aq})} \quad (2)$$

The distribution of the sample, represented by A^+ and AB in equations (1) and (2), will be governed by the concentration of the counter ion B^- .

The mechanism of reversed phase ion pair chromatography is thought to operate like ion-exchange. Cation amines present in the test solute are thought to pair with counter ions that can exchange with the ion pair anions adsorbed onto or into the hydrocarbon surface of the packing. The nature of the ion pair acid as well as the concentration of the pairing ion is important in controlling the degree of retention. Longer alkyl chains on the acid generally produce longer retention times and increased resolution.

Catecholamines were adsorbed from pre-acidified urine onto activated alumina. They were then eluted with acid, and further washed with ethyl acetate which was found to be effective in removing interfering compounds. Catecholamines are particularly unstable at

alkaline pH. Samples with pH >4.0 are not suitable for analysis and were not processed. Dihydroxybenzylamine (DHBA) is the most popular internal standard, since it is not present physiologically and elutes close to but separately from the catecholamines, and behaves in a similar manner to catecholamines during the extraction procedure. The catecholamines remain positively charged throughout the pH range (pH 2-8) compatible with reverse phase columns. Thus it is not possible to overcome their hydrophilicity by increasing pH to suppress their ionization. The solution to this problem is the addition of ion-pairing agents to the mobile phase e.g. sodium octyl sulphate (PIC B8). The combination of a hydrophobic side chain and negatively charged group has the effect, once equilibrium has been reached with the stationary phase, of selectively retarding the elution of the positively charged amines.

Alumina binding is maximal at pH 8.6 which causes a significant loss of analyte due to catecholamine oxidation at alkaline pH. Many agents are known to stabilise catecholamines including EDTA, sodium metabisulphite/EDTA, ascorbic acid, or ethyleneglycol bis (aminoethylether) tetra-acetic acid (EGTA). A combination of sodium metabisulphite and ascorbic acid at a final concentration of 2.8mmol/l afforded the necessary stability to the catecholamines. There appeared to be a relationship between internal standard recovery and urine concentration, a fact which was overcome by the dilution of the sample with 3mol/L Tris Buffer.

Method

Alumina was acid washed in 0.1N HCL and oven dried at 200°C before use. A stock combined standard contained 20mg each of noradrenaline and adrenaline, and 24.8mg of dopamine made up to a final volume of 1 litre with 0.1mol/L HCL. Dilutions of this were made to obtain working standards of various concentrations. A working standard containing 590nmol/L noradrenaline, 545nmol/L adrenaline and 750nmol/L dopamine was used for calibration purposes. The stock internal standard consisted of 31.7mg of DHBA made up to 1 litre with 0.1mol/L HCL. A suitable dilution of this corresponding to a value of 650nmol/L was used as working internal standard to be added to the samples during the course of the extraction procedure. The reducing agent, which was made freshly with each batch of analyses consisted of 1.9gm sodium metabisulphite and 1.76gm ascorbic acid in 100ml of water.

The mobile phase contained 15.6gm sodium dihydrogen orthophosphate, 40mg EDTA, and one vial of PIC B8 (the ion pairing reagent) is brought to 1 litre with polished water and adjusted if necessary to pH 3.5. The mobile phase is then filtered and degassed prior to introduction onto the column. The ion-pairing reagent available commercially is expensive, and the mobile phase was recycled to cut down on running costs. This does not affect the sensitivity or specificity of the assay. The 3mol/L tris buffer is maintained at pH 8.75 and contains 363gm Tris (hydroxymethyl) methylamine, 50gm EDTA in 1 litre of water. A barbitone buffer made up to 2 litres with water and adjusted to pH 8.5, contained 41.2gm sodium barbitone and 8ml of freshly prepared reducing agent.

Sample Preparation

Special glass-stoppered tubes capable of holding 12ml of solution, were made to specification for this assay. 4.5ml of standard, urine or control, followed by 0.2ml of freshly prepared reducing solution and 1ml of internal standard were placed in a glass tube and vortex mixed. This was followed by the addition of 4.5ml of Tris buffer which leaves the pH of the sample between 8.5 and 8.7. Alumina was then added and the tubes were tumble mixed for 20 minutes. This was followed by centrifugation and removal of the supernatant. The alumina containing the adsorbed catecholamines was then washed with 10ml of barbitone buffer pH 8.5, mixed for 5 minutes, centrifuged, and the buffer was removed and discarded. This was followed by elution of the catecholamines into 0.5ml of 0.5 N HCL. After centrifugation 0.4ml of the supernatant was added to a fresh tube containing 8ml ethyl acetate. This solution was vortex mixed for 60 seconds and the ethyl acetate layer discarded. 15 μ l of the lower aqueous layer was injected onto the column for separation and quantification. Calculation was by peak height ratios with absolute recovery (%) based on internal standard.

Chromatographic conditions

Column	:	u Bondapak C 10 μ m
		C / corasil 37 - 50 μ m (guard column)
Flow Rate	:	1.25ml / min
Electrochemical detector	:	Potential + 0.67 volts
		Filter 0.5
		Range 0 – 250 nA
		Offset 0 (set)

A 1/10 dilution of each sample was prepared for creatinine analysis which was carried out on a Hitachi 737 analyser by the Jaffe reaction.

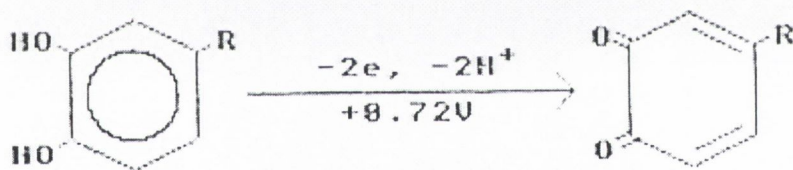
Lyphocheck I and II are used as controls in both the above methodologies. Performance in the United Kingdom External Quality Control Scheme (UKEQAS) for urinary catecholamines and metabolites has proved to be satisfactory.

Electrochemical detection of catecholamines

Electrochemical detectors oxidise catecholamines. The oxidation is driven by applying a positive potential. Electrons are transferred to the electrode, the resultant current being directly proportional to the number of molecules oxidized. The potential at which oxidation occurs depends on the ease with which the compound can be oxidized. Catecholamines oxidize at low potentials usually between +0.50 and +0.70 volts using standard glassy carbon electrodes. Amperometric detectors for catecholamines consist of carbon-paste or glassy carbon working electrodes. Carbon paste is composed of very fine particles of graphite and a binder mixed to the consistency of a semi-dry paste. The binder holds the particles of graphite together as an integral unit and should be chemically and physically inert. Paraffin oil and silicone grease have been proven

suitable for this purpose. The choice of the binder is crucial as acetonitrile which is a constituent of many mobile phases dissolves paraffin oil and therefore cannot be used with this binder. Glassy carbon electrodes have largely replaced these as general purpose electrodes for amperometric detectors. Glassy carbon is a hard, brittle solvent-impervious electrode material. Because it is 100% carbon the chemical and physical resistance of glassy carbon to all mobile phases are unequal. The carbon-paste electrode is capable of providing higher sensitivity than the glassy carbon electrode under optimum conditions.

In clinical studies, two main kinds of detectors are used, amperometric or coulometric with either single or dual electrode systems. The Waters 460 employs a three electrode amperometric cell consisting of a reference, a working and an auxiliary electrode. The potential is applied to the working electrode and is maintained, via a reference electrode, by passing the required current through the working and auxiliary electrodes. The electrochemical reaction and current measurement occur at the working electrode. Approximately 5% of the analyte is oxidized at the surface of these glassy carbon electrodes in these amperometric detectors. The voltage set at + 0.80 V gave maximum sensitivity for the metabolic compounds. The detector output was converted from analogue to digital by means of a Waters detector interface module and processed using the Waters 'Maxima' data handling package.



Legend – In the amperometric mode, the electrode is maintained at a constant operating potential. The operating potential drives the ortho-dihydroxy group of the catecholamine to undergo a loss of 2 electrons and 2 protons to yield an ortho-quinone. The electrons pass into the electrode and the resulting current is converted to a voltage, amplified and measured with an appropriate time base recorder or integrator. The current is directly proportional to the concentration of solute passing over the electrode. As oxidisable bands of solute pass the detector, the current rises and falls as a function of time to yield a typical chromatogram.

All electrochemical monitoring devices depend on Faraday's law:

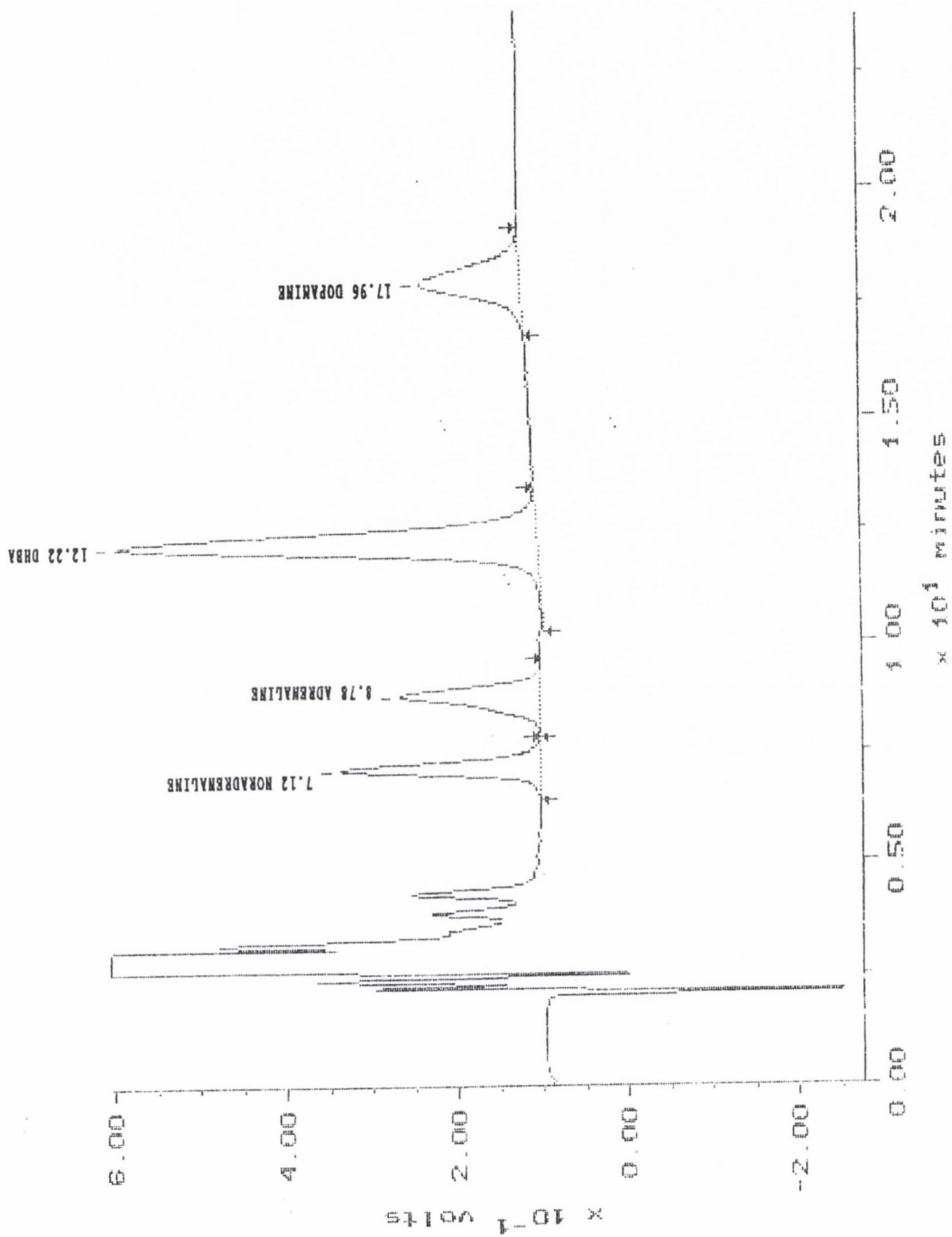
$$Q = nF \quad (3)$$

Where Q is the number of coulombs required to convert n moles of reactant to product, Z is the number of electrons per ion or molecule of reactant, and F is the Faraday constant (96,500 coulombs mol⁻¹).

When a detector electrolyses 100% of the analyte the detector is said to be coulometric. Dual electrode detectors have been constructed using two coulometric electrodes, two amperometric, and a coulometric and an amperometric electrode.

The laboratory switched to an ESA Coulochem 5100A detector due to a malfunction in the Waters 460. The ESA Coulochem 5100A comprises a high sensitivity dual electrode cell containing one high surface area coulometric electrode, combined with an amperometric electrode. This amperometric electrode is more efficient than the comparable Waters type oxidizing approximately 70% of the analyte. The combination of an enhanced response amperometric electrode with the capability of the coulometric electrode to screen interfering substances provides greater sensitivity. A high level of selectivity is further achieved by the inclusion of a conditioning cell which pretreats the sample prior to its introduction into the analysis cell.

Using the Waters 460 at a potential of +0.67 volts maximum sensitivity for the simultaneous analysis of noradrenaline, adrenaline, DHBA and dopamine was achieved. Even greater sensitivity for these analytes was achieved with the ESA Coulochem at the following potentials; detector 1, +0.05 volts; detector 2, -0.35volts; and conditioning cell potential +0.35 volts. Using the Waters 460 at the previously recommended potential of +0.67 volts and despite stepwise alterations in the applied potential between +0.64 and +0.80 volts together with systematic adjustments in the percentage components of the mobile phase, satisfactory simultaneous oxidation of the amine metabolites was not achieved. The clear separation peaks achieved with the Coulochem are shown in the figure below.



Legend: Normal Urinary Catecholamine Profile

The ESA Coulochem II Multi-Electrode Detector is currently in use. This detector includes a control module which contains two potentiostats which are connected to the cell via a cable. All commands can be entered via a computer terminal using the remote communication link incorporated into the Coulochem II. Both amperometric and

coulometric electrodes are designed for use with the Coulochem II. The coulometric flow cells used in the Coulochem II are designed so that the eluent flows through porous graphite electrodes rather than over the electrode as with other electrochemical detectors. When the Coulochem II uses coulometric cells, essentially all of the electroactive species are oxidized (or reduced). As a result, the detector provides higher sensitivity than when amperometric cells are used. The detector contains porous graphite electrodes. These electrodes have a very large surface area to ensure a coulometric response. The graphite electrode and small diameter tubing can act as an efficient filter for small particles, such as silica fines from the column and particulate matter in the sample. These particles tend to clog the cell and impede the flow of the mobile phase. To avoid clogging the cell, an inline filter containing a graphite filter element must precede each electrochemical cell in the system.

Each ESA analysis cell contains a screening electrode which reacts with 100% of interfering electroactive compounds. This reaction eliminates potentially interfering compounds. The dual electrode system coupled with the use of a conditioning cell placed between the column and the analysis cell enhances the selectivity and stability of the detector.

Retention times of Catecholamines

Catecholamine	Retention time in minutes
Noradrenaline	between 6 and 7 minutes
Adrenaline	between 7 and 8 minutes
Dopamine	from 12 minutes onwards
Dihydroxybenzylamine (DHBA)	between 9 and 12 minutes

When samples from babies were being processed where sterile disposable paediatric urine container bags had been used, it sometimes proved impossible to repeat samples due to low sample volume. This occurred infrequently in some concentrated urines which had very high dopamine values which had exceeded the detector scale. Some low adrenaline values were lost due to deterioration in the sensitivity of the assay for this analyte alone, and this was picked up by the internal control procedures and was partly related to the recycling of the mobile phase found necessary due to cost.

Appendix 1

This shows examples of the external quality assurance data from the laboratory which has been satisfactory over the past decade.

Assay Performance

Precision

Using commercially available control urine (*n* = 15 at each level), the within batch coefficients of variation (CVs) were, respectively, 9.5% at 21µmol/L and 4.9% at

82µmol/L for HMMA, 7.1% at 14µmol/L and 5.0% at 80µmol/L for HVA, 4.4% at 0.248µmol/L and 8.1% at 0.998µmol/L for noradrenaline, 9.3% at 0.073µmol/L and 3.4% at 0.292µmol/L for adrenaline, and 2.8% at 0.780µmol/L and 3.4% at 2.678µmol/L for dopamine. The between batch CVs, calculated over 30 consecutive procedures in a 12 month period were 15.0% at 20µmol/L and 6.3% at 80µmol/L for HMMA, 18.7% at 16µmol/L and 7.5% at 80µmol/L for HVA, 8.5% at 0.234µmol/L and 8.9% at 0.996µmol/L for noradrenaline and 7.4% at 0.698µmol/L and 10.3% at 2.549µmol/L for dopamine. Between batch CVs for adrenaline were 28.2% at 0.068µmol/L and 9.2% at 0.330µmol/L calculated from 24 assay procedures. The poor precision at low levels has been a problem with this assay. A column change and renewals of the mobile phase were carried out during the study period. The results were unaffected by the changes.

Recovery

Forty urines spiked with aqueous standard were used to determine recovery. The values for HMMA and HVA at 110µmol/L, noradrenaline at 0.295µmol/L, adrenaline at 0.115µmol/L, and dopamine at 0.325µmol/L were 93%, 98%, 92%, 95% and 88%, respectively.

Linearity

HMMA and HVA were linear to at least 220 and 180µmol/L, respectively. Noradrenaline was linear up to 2.0µmol/L, adrenaline to 2.6µmol/L and dopamine to 3.0µmol/L.

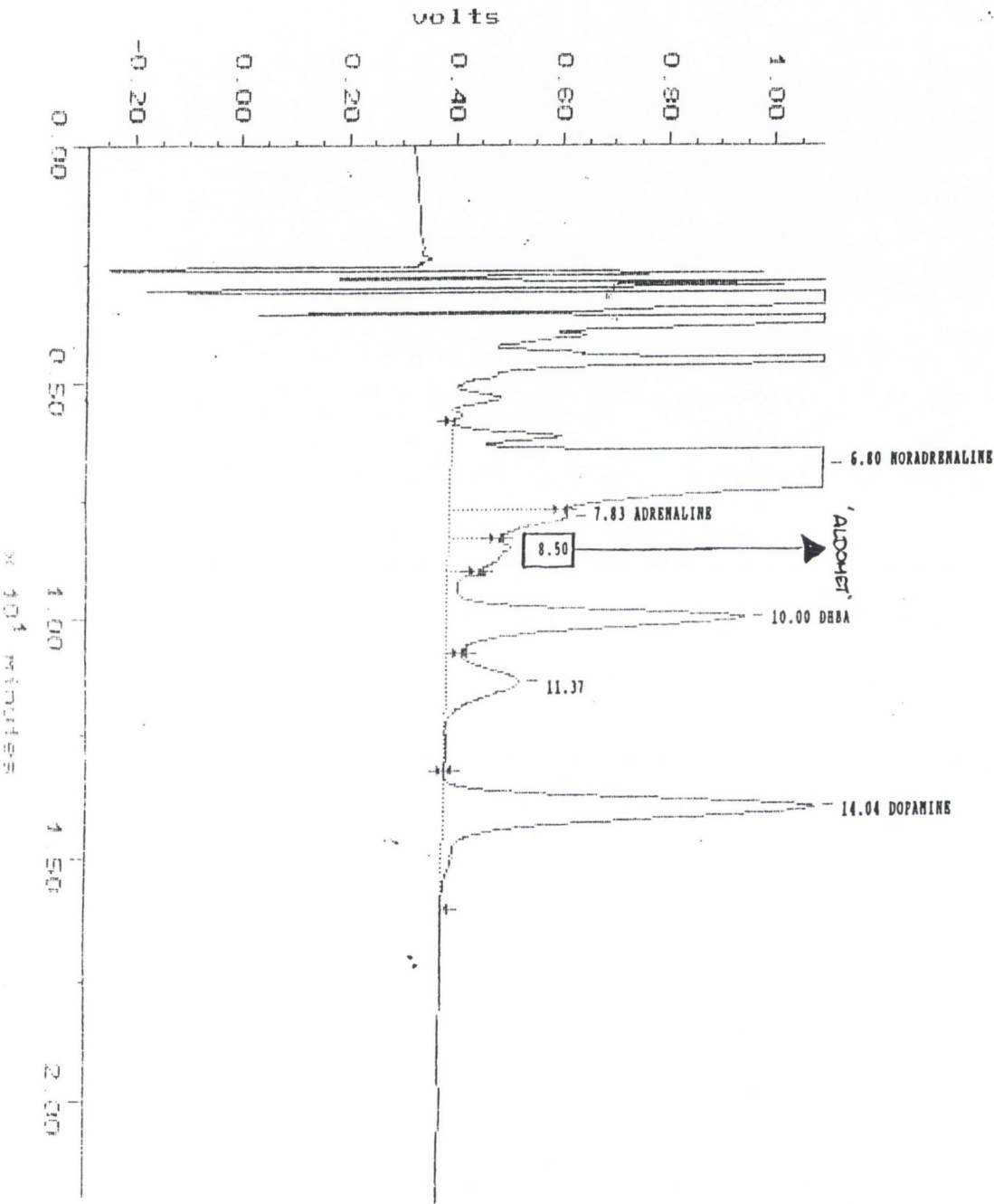
Common drug interferences in Urinary Catecholamine Method

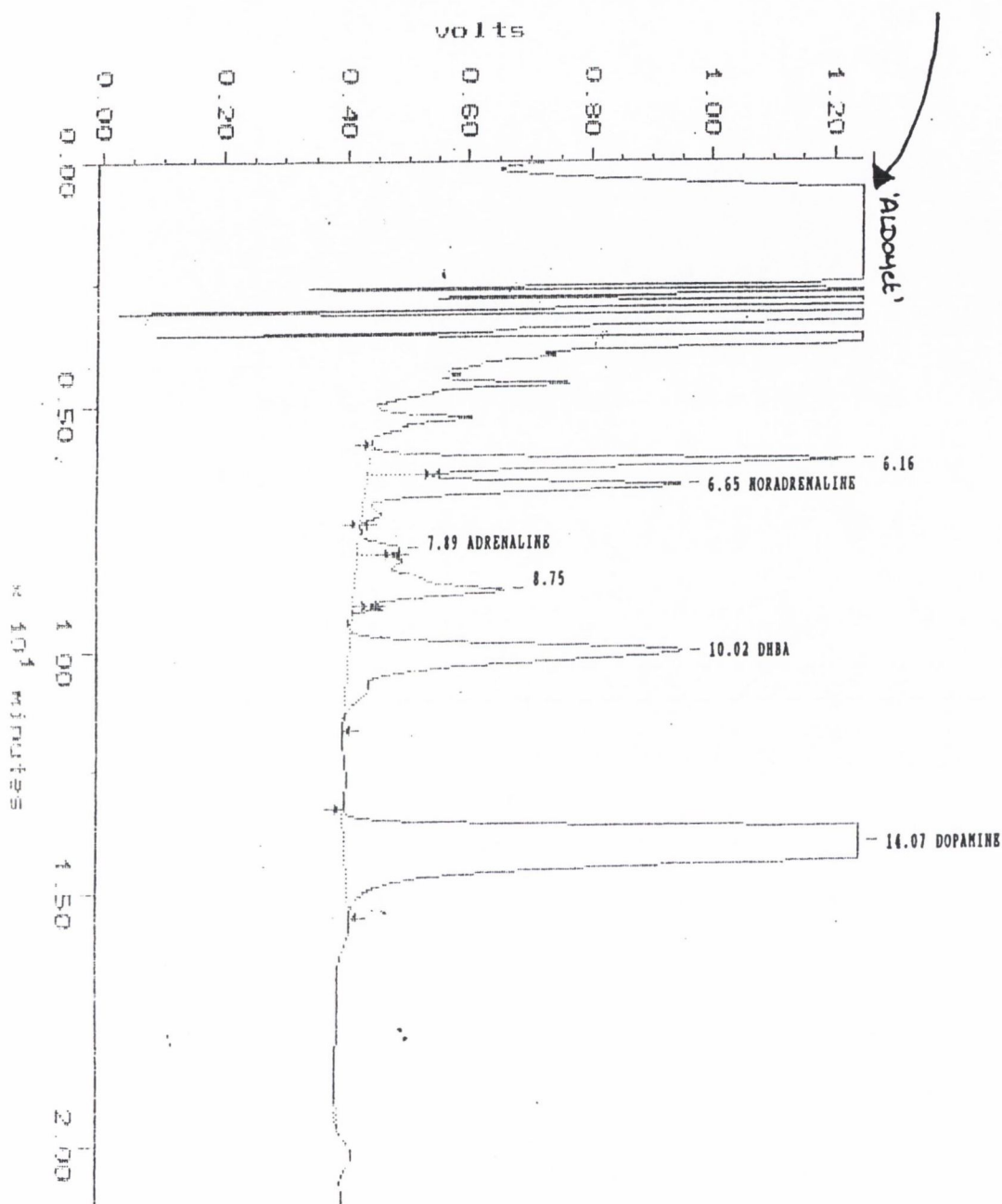
- | | |
|------------------------------|---|
| Alpha-methyl-dopa (Aldomet): | Peak with a retention time the same as adrenaline and another peak with twice the retention time of dopamine. |
| Labetalol (Trandate) : | Peak at the same retention time as DHBA. |
| Paracetamol : | A peak is found midway between adrenaline and DHBA internal standard. As the column ages, this may co-chromatogram with adrenaline. Free paracetamol produces a peak at shorter retention time. |
| Sinemet : | Peak at the same retention time as noradrenaline. |

*Sinemet is a combination of L-dopa and carbidopa

Examples of interferences follow.

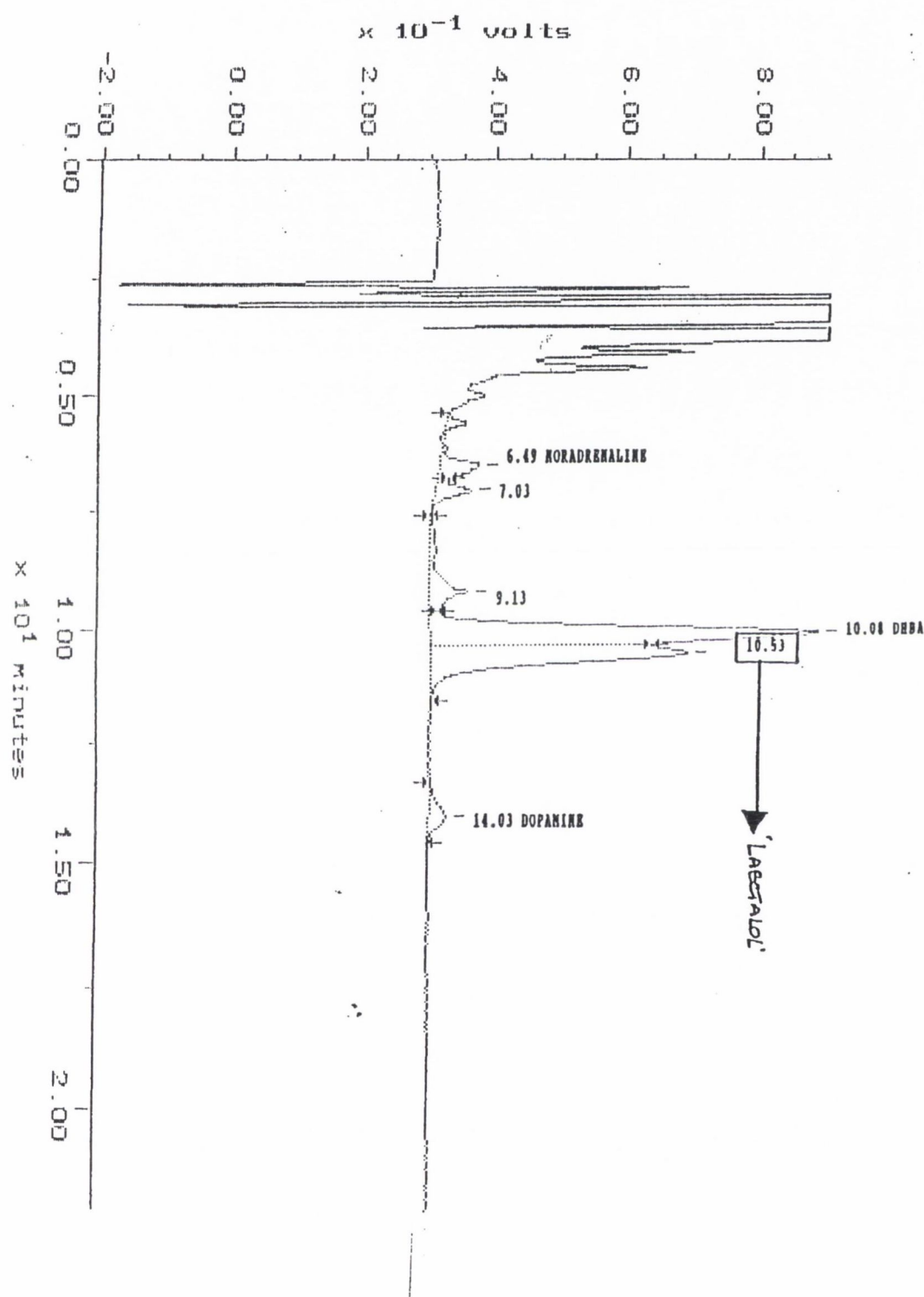
Chromatograms showing the effect of the presence of alpha methyl dopa (aldomet).



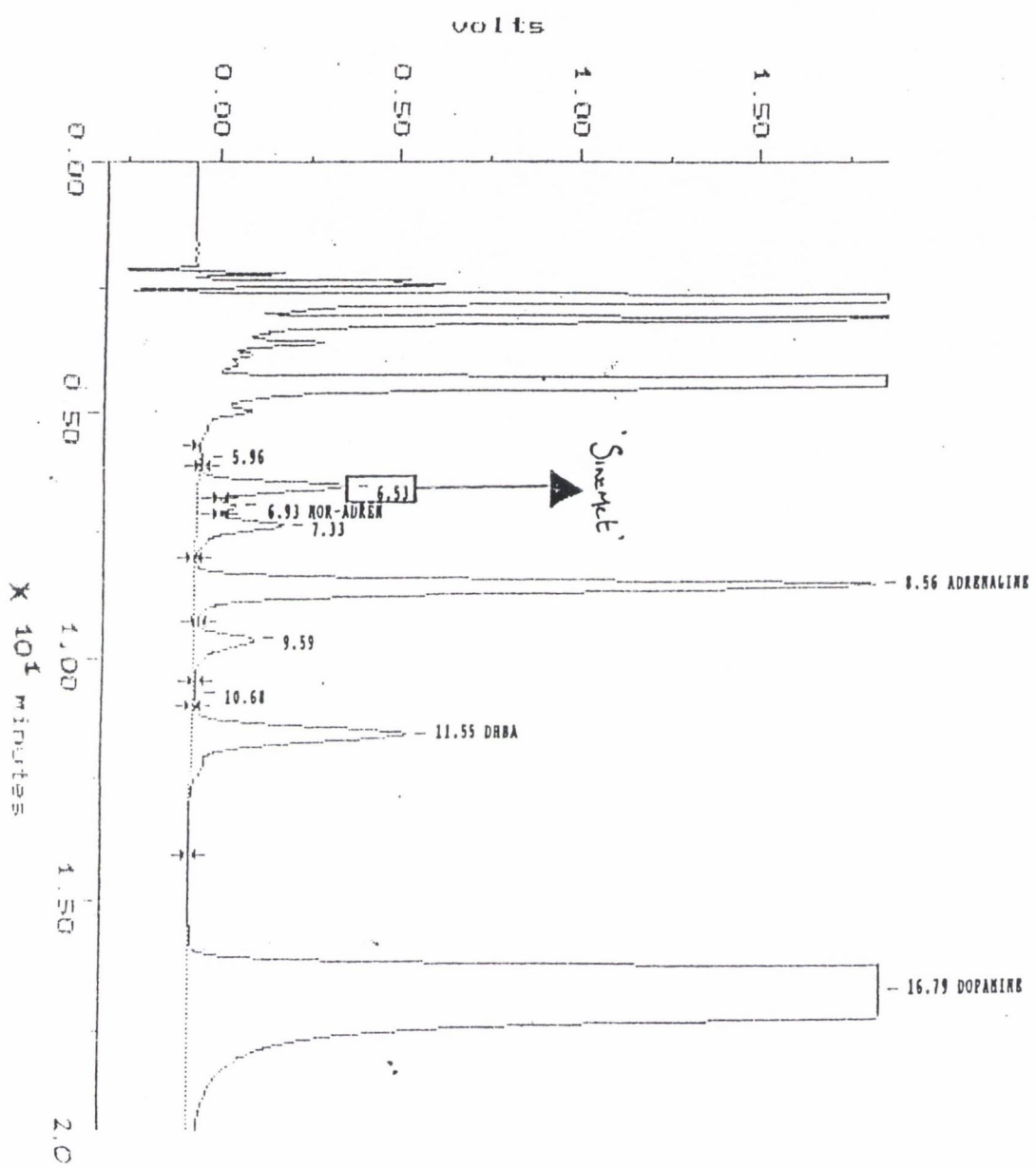


Legend: The "aldomet" present in the higher chromatogram at about 8.5 minutes follows with a second peak before the solvent front from the succeeding sample separation. This may interfere with the estimation of adrenaline. The first peak is thought to be due to the alpha methyl dopa metabolite α -methylnoradrenaline. Nifedipine may produce a peak which also runs very close to adrenaline (Dr N Lawson, East Birmingham Hospital, Personal Communication).

Chromatogram showing the effect of labetalol interference. The labetalol peak overlaps that of DHBA.



Sinemet may interfere with noradrenaline estimation and with dopamine.

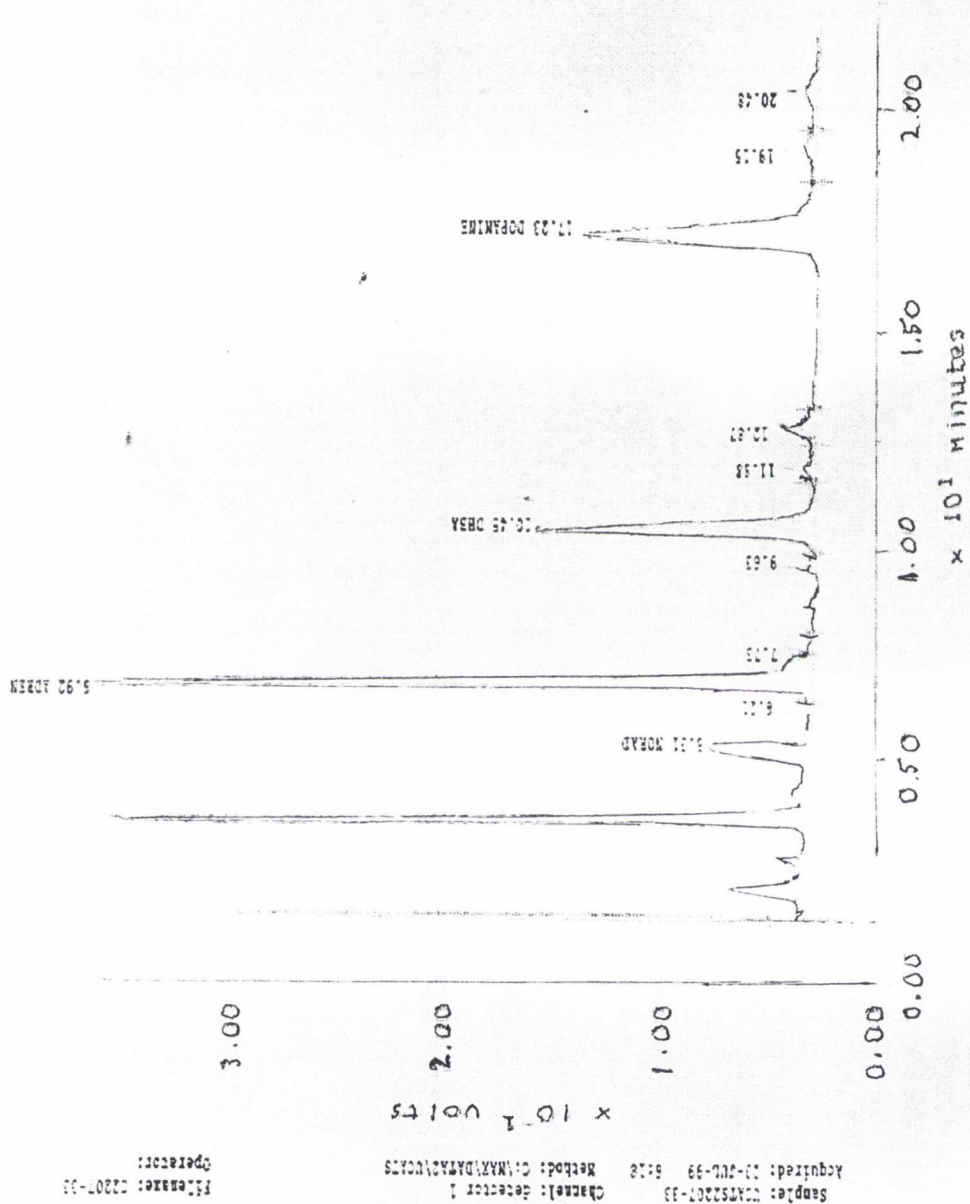


Paracetamol interference – a practical example.

A 68 year old woman presented with episodes of severe hypertension and episodes of headache and abdominal pain. She had no sweating attacks or palpitations and was treated with lisinopril.

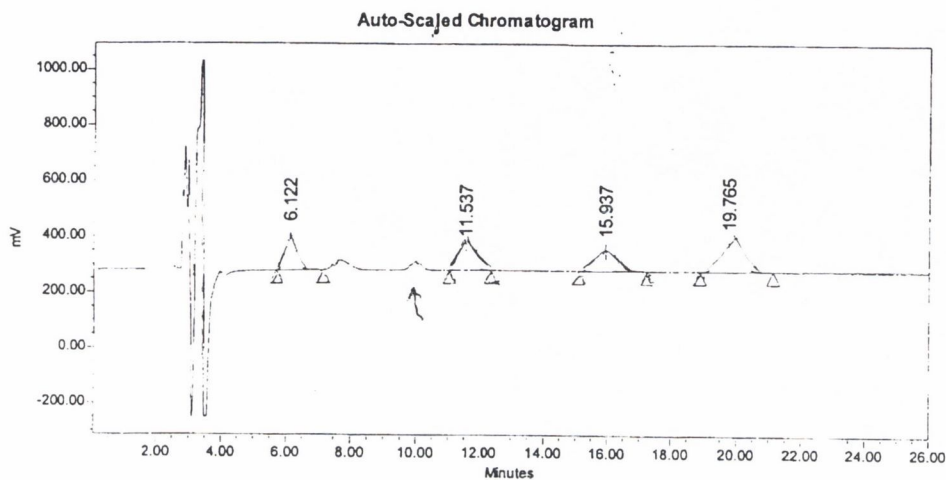
In 1994, a catecholamine screen was performed. The results were HMMA 18.6, HVA 17.1 pmol/L. In 1995, the tests were repeated on clinical grounds due to swings in blood pressure. The results were HMMA 36.0, HVA 17.0. All normal.

In 1999, she was rechecked due to persistence of her symptoms. Only lisinopril was listed as drug therapy on receipt of the specimen. The result was noradrenaline 230, adrenaline 3146 and dopamine 1544 $\mu\text{mol/L}$. The chromatogram is shown on the following page.



On reviewing the case with the clinician, paracetamol interference was raised as an explanation. The urine paracetamol was 17 µg/ml using the Dade-Behring EMIT assay in a Cobas Viva analyser.

The sample was then run on the ASTED. The chromatogram is shown below.

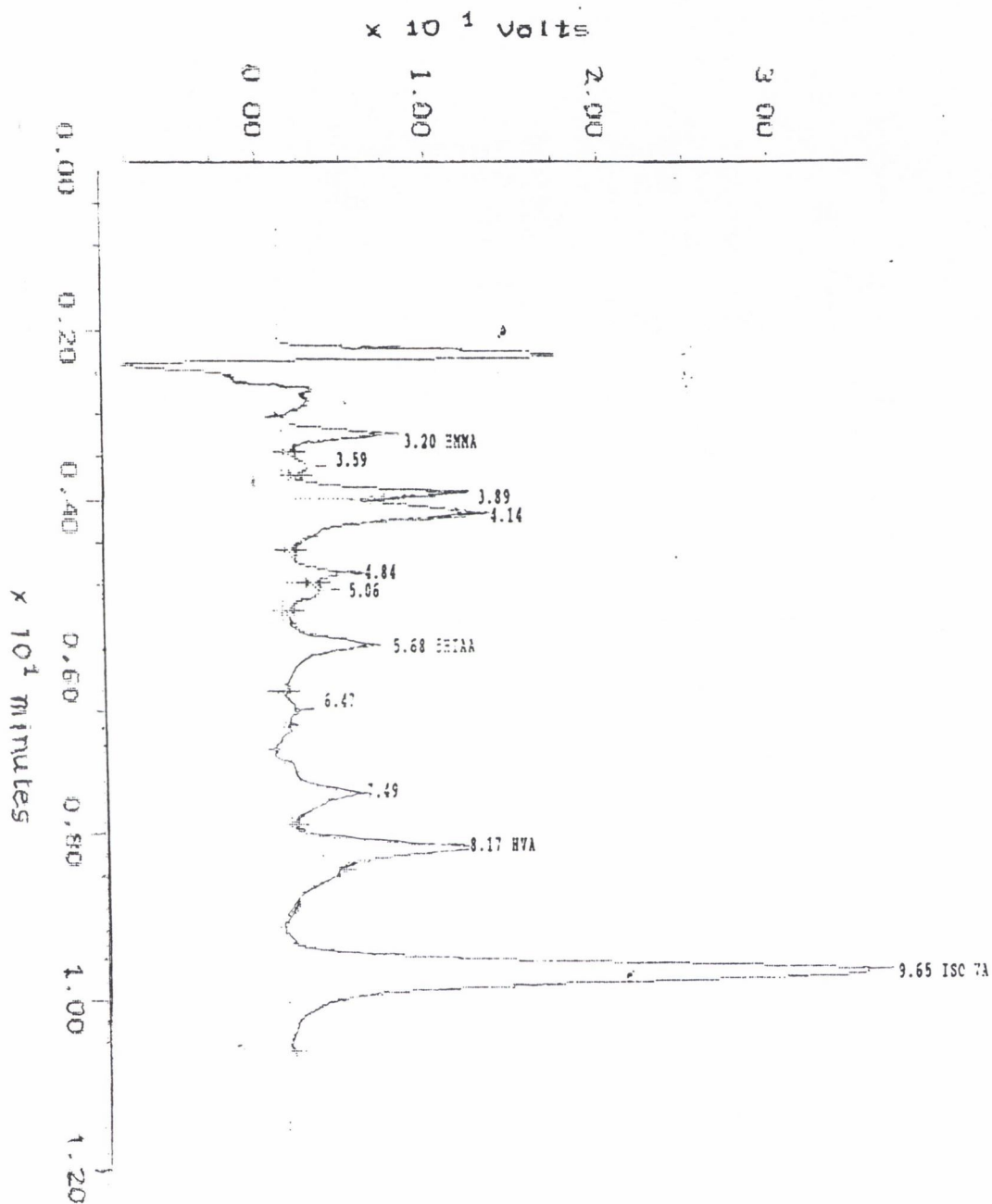


6.122 is noradrenaline, 11.537 is the internal standard DHBA, 15.937 is dopamine and 19.765 is an unknown peak. The adrenaline is the un-numbered peak at 10.0 minutes. This indicates a very low adrenaline peak in sharp contrast to the chromatogram from the same sample shown on the previous page.

The metabolites are shown in the chromatogram from the ASTED on the following page.

Sample: EMMA0101-29 Channel: ESA 5200A
Acquired: 04-AUG-99 23:09 Method: C:\MAX\DATA\UREMMA

Filename: H0102-29
Operator:

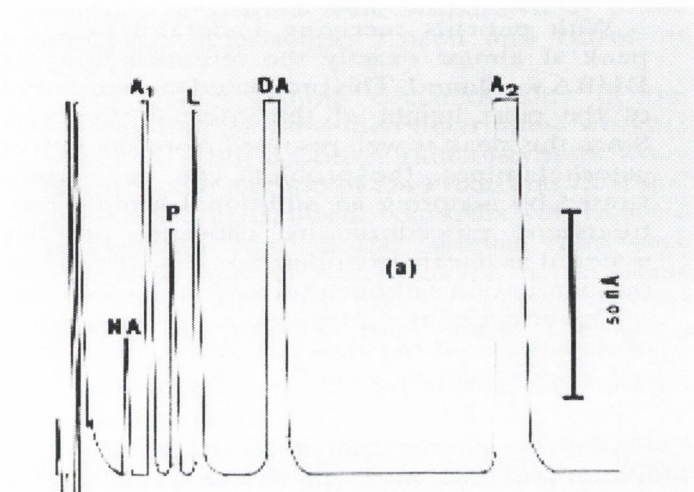


The peak at 4.14 on page 74 is paracetamol.

A repeat specimen was collected two days later. The results were noradrenaline 134, adrenaline 26, dopamine 1128 $\mu\text{mol/L}$ – all normal. The urine was checked for paracetamol and none was detected.

In conclusion, it is important to ensure that the apparently raised adrenaline due to drug interference is not reported as a true value as expensive imaging scans would immediately be initiated.

Chromatographic representation of paracetamol, labetalol and alpha methyl dopa (aldomet) interferences ³.



- NA = Noradrenaline
- A₁ and A₂ = Alpha methyl dopa (aldomet) associated peaks
- P = Paracetamol associated peak
- L = Labetalol associated peak
- DA = Dopamine

The peaks for alpha methyl dopa and labetalol have been superimposed on those for adrenaline and DHBA. Patients taking labetalol prevent the accurate estimation of catecholamines using this method. The problem can be addressed by the adoption of additional sample pre-treatment procedures or by the choice of a different internal standard, or by estimation without internal standardisation of that particular sample. Changing the patients medication may also be an option.

References:

1. Tietz Textbook of Clinical Chemistry. Section 11. High-performance liquid chromatography. Analytical techniques and instrumentation. 3rd Ed, Saunders, Philadelphia, 1999; P184- 92.
2. Davidson DF. Simultaneous assay for urinary 4-hydroxy-3-methoxy-mandelic acid, 5-hydroxyindoleacetic acid and homovanillic acid by isocratic HPLC with electrochemical detection. Ann Clin Biochem 1989;26:137-43.
3. Davidson DF. Urinary catecholamine assay by HPLC: in vitro interference by some drugs. Ann Clin Biochem 1988;25:583-4.

Materials

All water used was purified by reverse osmosis with further polishing on a Millipore (Milli Q) Water purification system.

Chemicals were obtained from:-

- a) Sigma Chemical Company Ltd, Fancy Road, Poole, Dorset, BH 17 7NH, England.
- b) B.D.H. Chemicals Ltd, Broom Road, Poole, Dorset, BH12 7NN, England.
- c) Bio-Rad Laboratories Ltd, Caxon Way, Watford Business Park, Watford, Hertfordshire, WD1 8RP.
- d) ESA, Inc 45, Wiggins Avunue, Bedford, MA 01730.
- e) Waters Chromatography Division, Millipore Corporation, 34 Maple Street, Milford, Massachusetts 01757, USA.

Reagents for urinary catecholamines extraction and analysis by HPLC-ECD

- | | | |
|-----|--|---------------------|
| 1. | Aluminium oxide | BDH Cat No 15141 |
| 2. | Ethyl acetate | Labscan HPLC grade |
| 3. | Sodium Barbitone | BDH Cat No 10365 |
| 4. | Hydrochloric Acid (convol) | BDH Cat No 18007 |
| 5. | Sodium metabisulphite | BDH Cat No 30180 |
| 6. | Ascorbic Acid | BDH Cat No 10303 |
| 7. | Sodium dihydrogen orthophosphate dihydrate | BDH Cat No 10245 |
| 8. | Ethylene-diamine tetra acetic acid (EDTA) | BDH Cat No 28026 |
| 9. | Pic B8 octane sulphate | Waters Cat No 85142 |
| 10. | Tris (hydroxymethyl) methylamine – Standards | BDH Cat No 10315 |

11.	Noradrenaline	Sigma Cat No A7257
12.	Adrenaline	Sigma Cat No E4250
13.	Dopamine	Sigma Cat No H8502
14.	Dihydroxybenzylamine	Sigma Cat No D7012

Reagents for the HMMA, HVA & 5-HIAA extraction and analysis by HPLC – ECD

All reagents are Analar grade.

1.	Ethanol	BDH Cat No 45101
2.	Ethyl Acetate	Labscan HPLC grade
3.	Boric Acid	BDH Cat No 5111 4 DR
4.	Orthophosphoric acid	BDH Cat No 45010 (Aristar grade)
5.	Potassium dihydrogen orthophosphate	BDH Cat No 10203
6.	Ethylene-diamine tetra-acetic acid	BDH Cat No 10093
7.	Methanol	Labscan HPLC grade
8.	Hydrochloric Acid	BDH Cat No 10125
9.	Disodium hydrogen orthophosphate	BDH Cat No 30157

Standards

10.	HMMA	Sigma Cat No H0131
11.	5-HIAA	Sigma Cat No H8876
12.	HVA	Sigma Cat No H1252
13.	Isovanillic Acid	Sigma Cat No I5878

For both the above methods Lyphocheck I and Lyphocheck II were used as urine controls and were obtained from Bio-Rad Laboratories.

Equipment

HPLC Equipment:-

Pump	: Waters Associates Model 6000A solvent delivery system.
Injector	: Waters Intelligent Sample Processor (WISP)
Detector	: (a) Waters Associates Amperometric Detector Model 460 (b) ESA Coulochem Model 5100A with series analytical cell, Model 5011 and conditioning cell Model 5021.
Data Handling:	Waters Associates IBM compatible chromatographic processing package - Maxima.
Columns	: (a) u Bondapak C, 30cm, 10µm particle size Waters Cat No 27324 (b) Guard Column, Waters Associates Cat No 84550 packing material C / corasil 37-50 µm Cat No 27248. (c) ODS 5µm, 150 x 4.6mm, Bio-Rad Cat No 195-5028.

Solvent clarification

- Apparatus : (a) Solvent clarification glassware without vacuum pump, Waters Associates - Cat No 85124.
(b) HA filters 0.45 and 0.22 μ m, Waters Cat No's HATF 04700 and GVWP 04700.
- Gases : Helium is required for solvent degassing, and a supply of compressed air is necessary for WISP function. Other equipment used was standard laboratory glassware, balances and stirrers. A Radiometer pH M61 laboratory pH Meter was used for estimation of pH of buffers and of patient samples.

Automated sequential trace enrichment of dialysates (ASTED) for sample preparation for reversed phase ion pair HPLC for catecholamines and metabolites

The ASTED process for catecholamine analysis is a combination of dialysis followed by trace enrichment and then injection onto the HPLC system. By adding a cartesian robotic sample handling system to the ASTED, automation of sample clean-up and HPLC analysis is achieved. In outline, the sample is dialysed to remove protein and other macromolecules. The catecholamines are then trace enriched onto a strong cation exchange resin column, the trace enrichment cartridge (TEC) with phosphate buffer passing over. The ASTED.XL uses two rheodyne valves and two dilutors. The programme then causes the enriched dialysate to be moved to the HPLC system via a valve. Acid is used to remove the catecholamines from the TEC and then they are injected on to the HPLC column and separated by reverse phase ion-pair chromatography with coulometric detection of the eluate analytes. The TEC and dialyser are regenerated for subsequent injections.

The hardware used is a Digital Venturis FX using Windows 95. The HPLC system is the Waters 600E with an ESA Coulochem 11.

The software used is 722 version 2 for ASTED™ XL from Gilson. The software used to drive the HPLC system is Waters Millenium r32 chromatography manager software, version 3.05 workstation and client/server.

The Decade amperometric detector (ANTEC Leyden, Holland) was used initially but the sensitivity was found to be less than that achievable with the Coulochem. To eliminate air contamination proved a particular problem and helium had to be bubbled through the mobile phase reservoir for about 30 minutes. This caused some loss of methanol which affected the retention times.

Reagents

1. Mobile phase

16.5 g diammonium hydrogen orthophosphate (BDH cat no 10025)

0.093 g disodium EDTA (BDH cat no 10093)

0.90 g heptanesulphonic acid sodium salt (SIGMA cat no H2766) (ion pair reagent)

Dissolve in approximately 900 ml deionised water.

Add 2ml HPLC grade methanol (pH approx 8 at this stage)

Adjust pH to 3.4 with conc orthophosphoric acid (approx 9ml) (BDH cat no 101173)

Make up to 1 litre with deionised water.

Filter with type HA filters and degas by sonication or helium (currently used) before use.

Prepare this reagent monthly or more frequently if required.

2. 0.1 M HCL (100 mmol/L)

8.6 ml conc HCl up to 1 litre with deionised water

3. (a) Stock catecholamine standard (10 mmol/L)

319.3 mg noradrenaline bitartrate
333.3 mg adrenaline bitartate
189.6 mg dopamine hydrochloride
219.7 mg normetanephrine hydrochloride
233.7 mg metanephrine hydrochloride

Dissolve in 100ml 0.1M HCl Store in fridge at 4 C.

(b) Intermediate catecholamine standard (100 µmol/L)

1 ml stock standard up to 100 ml with 0.1 M HCl (solution 2)
Prepare fresh before use and discard remainder.

(c) Working catecholamine standard (1000 nmol/L)

10 ml intermediate standard up to 1 litre with 0.1 M HCl (solution 2)

4 (a) 1M Sulphuric acid

Reagent C

54 ml conc sulphuric acid up to 1 litre with deionised water.
Always add acid slowly to water to avoid overheating.

(b) 50 mmol/L Sulphuric acid

25 ml 1M Sulphuric acid (reagent C) made up to 500 ml with deionised water.
Store in fridge at 4 c. Stable for 6 months.

(c) 10 mmol/L Sulphuric acid

5 ml 1 M. sulphuric acid (reagent C) made up to 500 ml with deionised water. Store in fridge at 4 c. Stable for 6 months.

5. Ammonium phosphate buffer

Reagent B.

132 g diammonium hydrogen phosphate.
Dissolve in approximately 1600 ml deionised water
pH to 8.3 with orthophosphoric acid.
Make up to 2 litres with deionised water.
Store in fridge at 4 c. Stable for 3 months.

6. Priming solvent for dilutor 10 mmol/L ammonium phosphate.

20 ml reagent B made up to 1 litre with deionised water.
Filter with HA filter (0.45 µm pore size) before use.

7. (a) stock internal standard

66 mg 3.4 dihydroxybenzylamine
Make up to 50 ml with 50 mmol/L sulphuric acid.
Store at 4 c. Stable for 3 months.

(b) Working internal standard

Reagent A

0.1 ml stock internal standard made up to 50 ml with 10 mmol/L sulphuric acid.

8. Diluent

Reagent D

0.9 NaCl made up in 0.1 N HCl

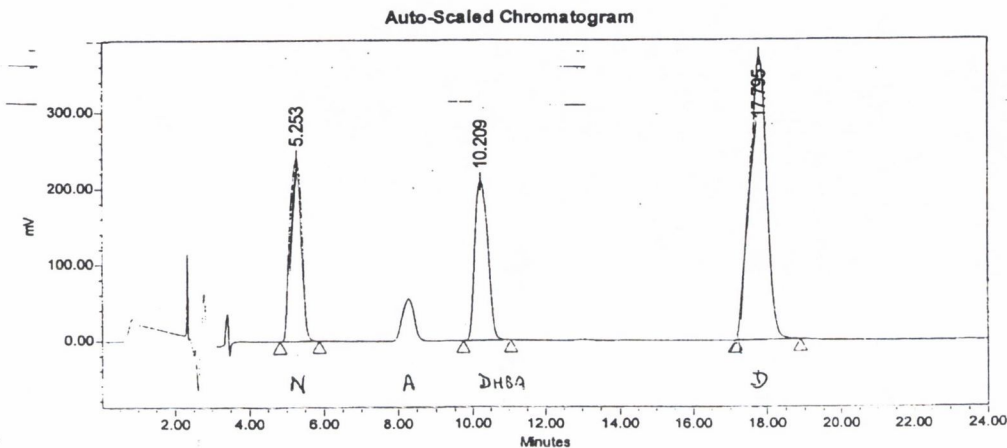
ASTED sample preparation for Catecholamines

1. Initializing
2. Rinse needle with priming solution – dilute buffer B –20 ml in 1 litre
3. Rinse injection port 500µl priming solution (dilute ammonium phosphate buffer)
4. Conditioning TEC 200µl C (1M sulphuric acid)
5. Conditioning TEC 1000µl priming solution
6. Aspirating 25µl from A (DHBA)
7. Aspirating 55µl B (Buffer)
8. Aspirating 200µl from sample
9. Dispense into result tube
10. Mixing result tube with 200µl priming solution
11. Aspirating 200µl from result tube
12. TEC enrichment with 200µl of result via dialysis
13. Aspirating 500µl from reservoir 1 M sulphuric acid
14. TEC washing with 500µl of dilute buffer B.
15. Inject

To get good separation, 15 ml of methanol was added to the mobile phase and the flow rate was set at 0.5 ml per minute. However, there were problems with the working standard. The first chromatogram gave good separation of the commercial standard which is used in the routine alumina extraction method described earlier and the chromatogram details are shown below. This working combined standard is mixture of pure standard for noradrenaline, adrenaline and dopamine, for example noradrenaline comes as a 'levophed' 1 mg for injection of noradrenaline base concentration 1 mg/ml as acid tartrate and 2 ml sodium bisulphite (Winthrop Sanof 2840315). There is a 20 minute constant gap between samples as then go through the system. Surprisingly, the second and subsequent chromatograms showed two extra peaks (P1 and P2) which were constant. This may have been due to an impurity in the standard eluting at 50 and 58 minutes. This did not arise with the alumina method and caused us to adjust the ASTED method. The chromatograms which follow in figures 1 and 2 are those using the 'levophed' 1000 nmol/L of each constituent as the working

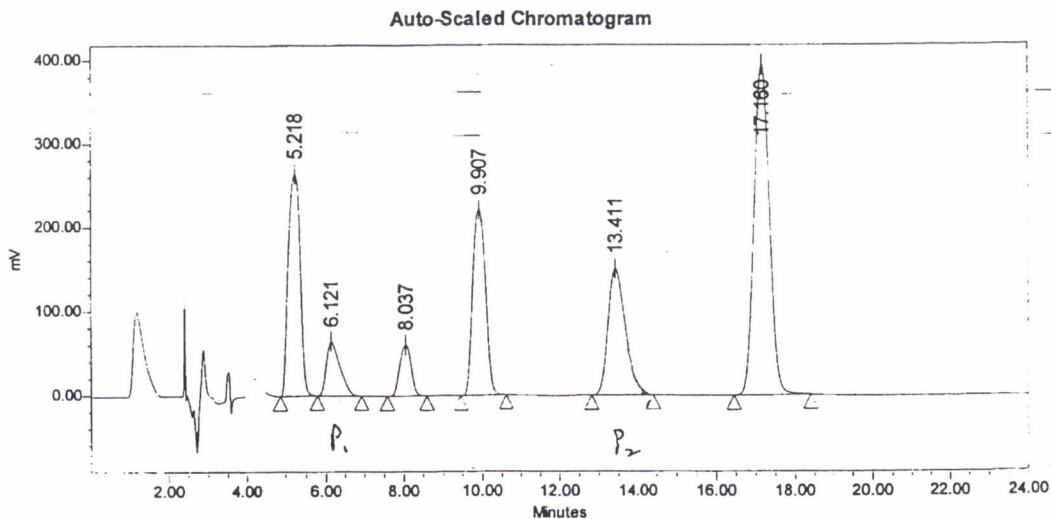
catecholamine standard. The chromatograms in figures 3,4 and 5 are of free catecholamine and metabolites standards 1000 nmol/L for each, made up from pure chemicals with the introduction of a diluent (0.9% NaCl in 0.1N HCl) and a reduction in the organic phase.

Figure 1



First chromatogram of the run showing noradrenaline (N) adrenaline (A), the internal standard dihydroxybenzylamine and dopamine.

Figure 2

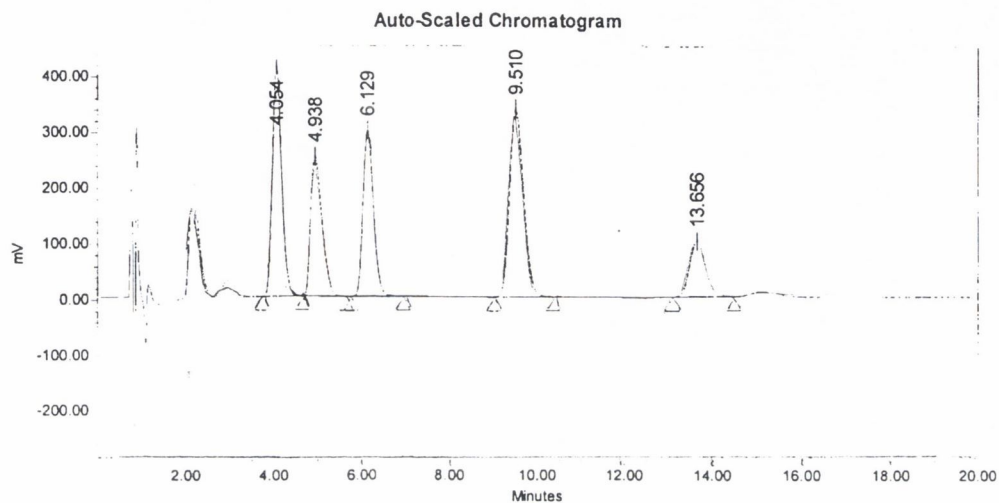


The second and subsequent chromatograms showing unexplained peaks at P1 and P2.

The volume of methanol that was added to the mobile phase was reduced from 15 ml to 2 ml and the flow rate was increased to 2 ml/min. The rest of the details were PSI 4000, sample volume 80 μ l, DHBA 10 μ l, Reagent D (diluent) 100 μ l, guard column +360 mv, E1 – 100 mv, E2 – 350 mv. We increased the acid wash volume for the

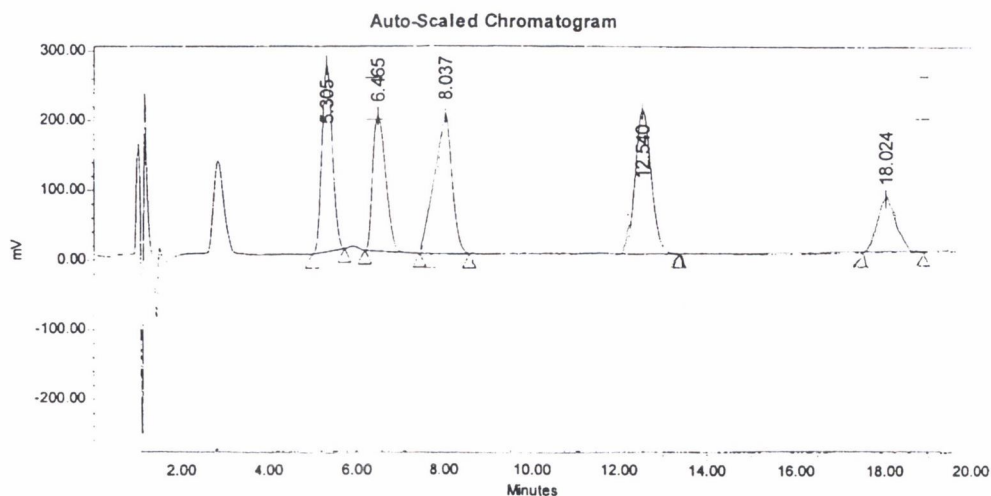
TEC from 500 μ l to 1000 μ l and repeated the wash prior to the introduction of the second and subsequent specimens. This resulted in the retention times shown in figure 3 below. The retention times in minutes were noradrenaline 2.1, adrenaline 4.05, dihydroxybenzylamine 4.94, normetanephine 6.13, dopamine 9.51 and metanephine 13.66. The constituent of the second small peak is unknown.

Figure 3



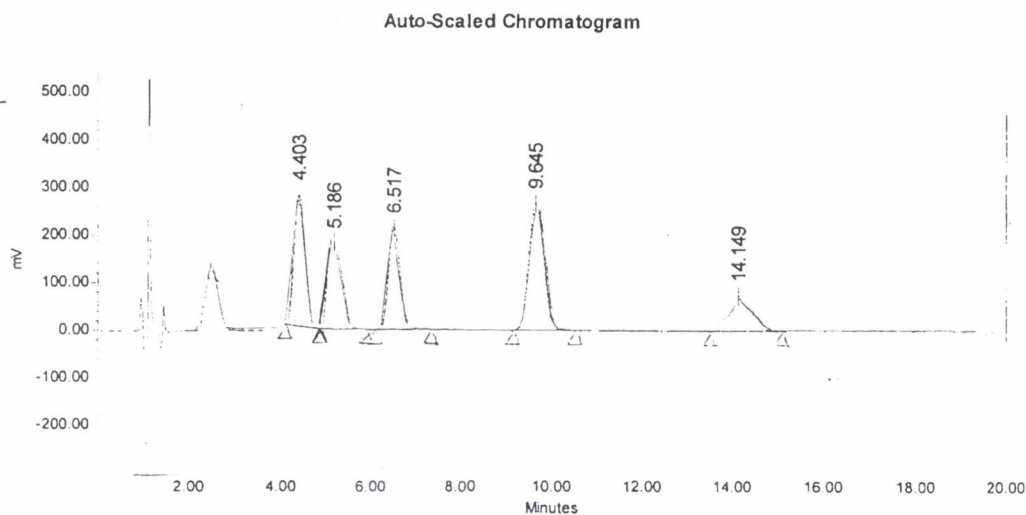
Because the noradrenaline peak was eluting too close to the solvent front, the flow rate was reduced to 1.5 ml/min, PSI 1360, Sample volume unchanged at 80 μ l, DHBA 10 μ l, Reagent D 100 μ l, guard +360 mv, E1 – 100 mv, E2 –350 mv. This altered the retention times as shown in Figure 4 below. Noradrenaline 2.75, adrenaline 5.3, DHBA 6.46, normetanephine 8.04, dopamine 12.54 and metanephine 18.02 in minutes.

Figure 4



Using the same mobile phase and the same working multi-standard, the analytical conditions were set as follows; flow rate 1.5 ml/minute, sample volume 80 µl, DHBA 10 µl, reagent D 100 µl, guard column +360 mvolts, E1 -50 mv, E2 -350 mv. Repeated chromatograms showed no interfering peaks and Figure 5 below is a representative sample. The retention times in minutes are noradrenaline 2.5, adrenaline 4.40, DHBA 5.1, normetanephine 6.7, dopamine 9.64, metanephine 14.15.

Figure 5



Paracetamol interference elimination

This is a problem with the alumina extraction method as shown earlier in this chapter. Figure 6A below shows a large adrenaline peak on a sample using the alumina extraction method. The urine paracetamol was 3 mg/L. Another aliquot of the sample was run on the Asted and the chromatogram was normal. This is shown in figure 6B. Paracetamol did not interfere. The molecular weight of paracetamol, is 151. It is likely to be removed by the TEC.

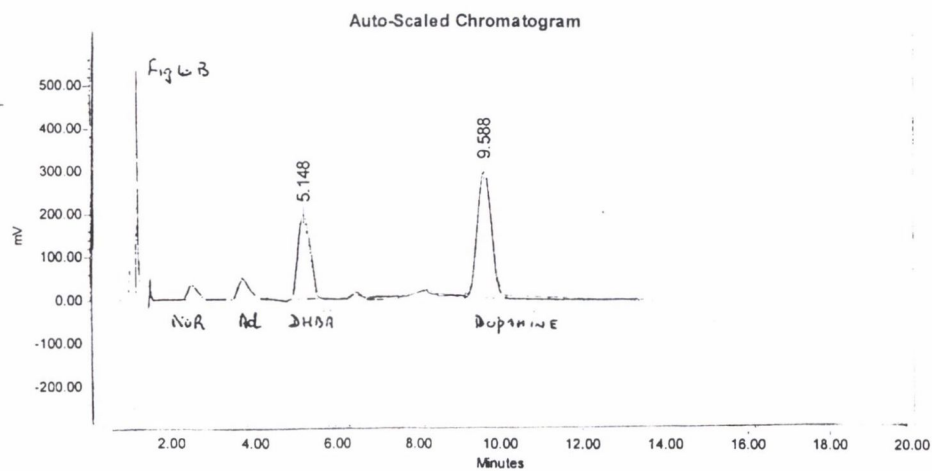
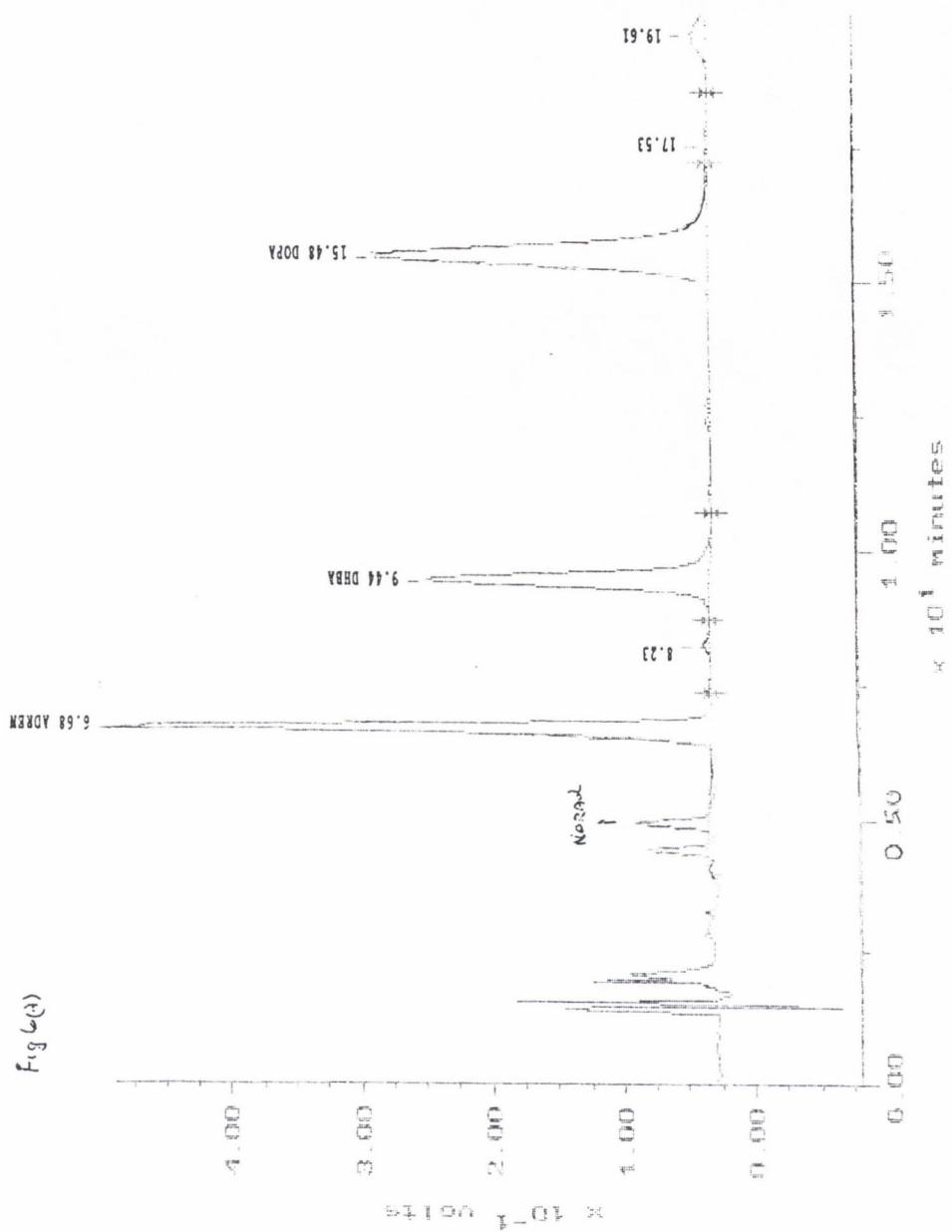
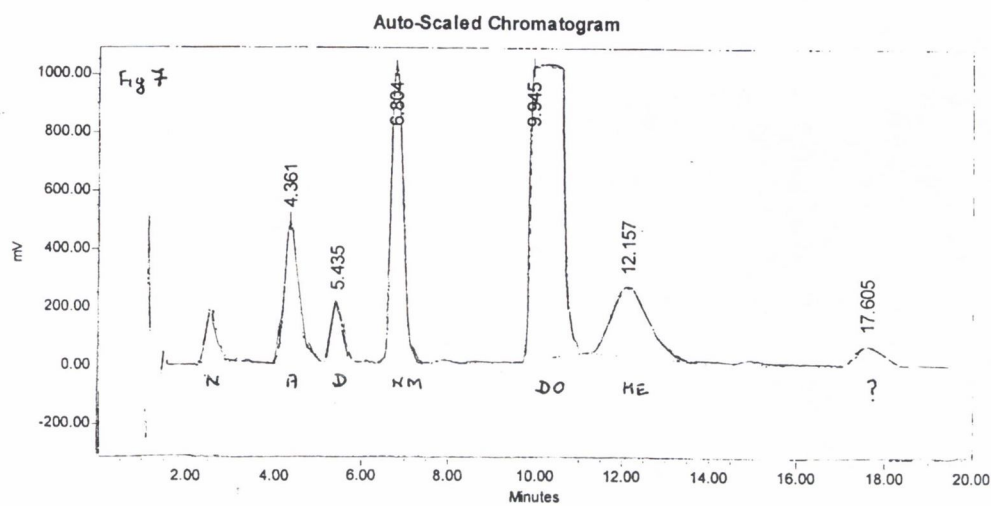


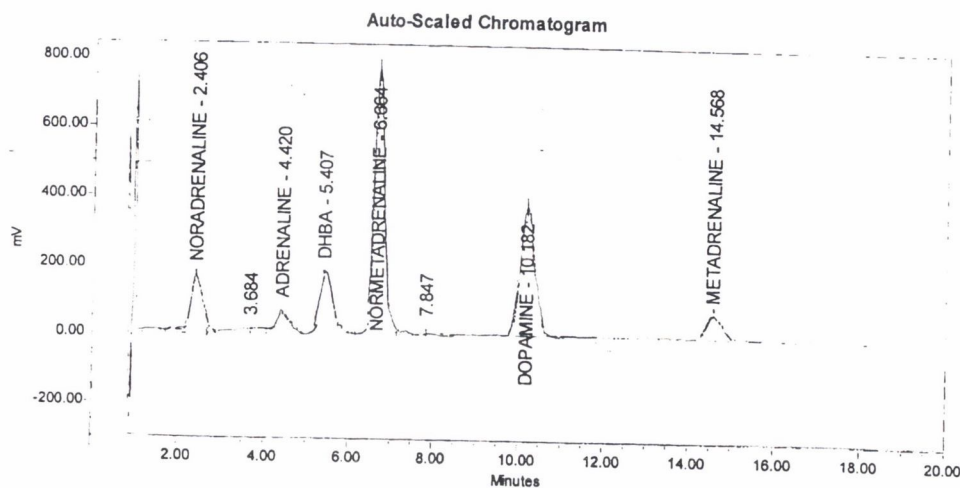
Figure 7 below shows a chromatogram with high levels of metanephrine and normetanephrine in a patient with neuroblastoma. The peaks shown are indicated N = noradrenaline, A = adrenaline, D = DHBA, NM = normetanephrine, DO = dopamine, ME = metanephrine and the peak at 17.605 min is unknown.



Replacement orthophosphoric acid for the mobile phase was purchased and had the effect of lowering the background noise on the electrochemical detectors. The settings on the Coulochem 11 were also altered to examine the effects on the output. Settings used were E₂ at -320 mv; i-38.4 nA : R2 500 nA : Ch2 0%. And E₂ -380 mv, I-13.7 na R2 500 na: CH2 0%. The background current decreased tenfold and we proceeded as below.

Figure 8 below shows a chromatogram of free analytes in a patient with a phaeochromocytoma.

The settings for Figures 8 and 9 are:- the flow rate was 1.8ml/min, the sample volume was 80 µl, DHBA 10µl, Reagent D 100 µl, guard column 320 mv, E1 at -100 mv, E2 at -320 mv and the range was 500mA.



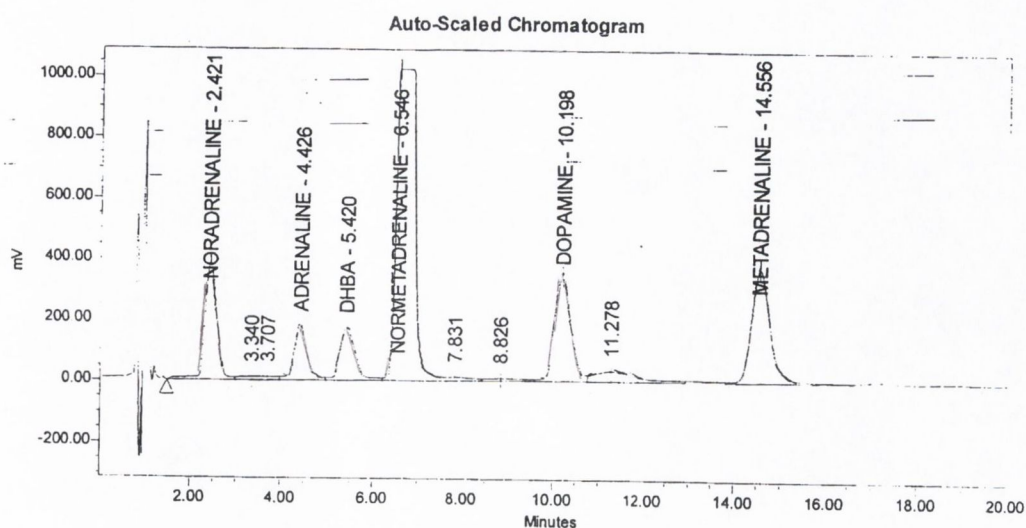


Figure 9 above is the chromatogram of total metanephrine and normetanephrine after hydrolysis of metanephrines, which are conjugated to sulphates and glucuronide, by boiling.

Using a standard made up with 0.1N HCl with 1000 nmol/L of normetanephrine and 1000 nmol/L of metanephrine, the within batch cv was 6.9% and 4.7% respectively, $n = 18$. Recovery was estimated by standard addition using a 500 nmol/L standard for normetanephrine and metanephrine. The recovery values for normetanephrine at 6279 nmol/L and metanephrine at 2008 nmol/L were 91% and 92% respectively.

Appendix 1

Quality Assurance

The External Quality Assurance from the UK NEQAS for urine catecholamines and metabolites from 1992 shows a varying performance with intermittent problems of bias both positive and negative. There is a trade-off between the duration of use of the columns before replacement, the age of the detector electrodes before replacement and the duration of re-circulation of the mobile phase. Cost is the major consideration in balancing clinical utility with scientific perfection.

The NEQAS report summaries at intervals from November 1996 to August 1998 are shown below as is the report of NEQAS distribution number 47 for noradrenaline and adrenaline in April 1998. These confirm the validity of the analytical work over these periods.

The Lypchocek (1) and (2), normal and abnormal respectively, are urine based quality control materials used in internal quality assurance procedures. These are manufactured by Bio-Rad and values are ascribed by the reagent manufacturer from extensive replicate analyses performed by the manufacturer and by independent laboratories, using manufacturer supported reagents and a representative sample of the control lot. A recent lot distribution with values using Bio-Rad HPLC test reversed phase are shown below. The values listed below are the stated means and the acceptable ranges for that batch number.

	LYPHOCHEK 1		LYPHOCHEK 2	
	Lot 62031		Lot 62032	
Noradrenaline	171	(130 – 213)	491	(414 – 567)
Adrenaline	55	(33 – 76)	322	(257-388)
Dopamine	424	(327 – 522)	1427	(1136 – 1711)
HMMA	16.0	(13 – 19)	71.0	(57 – 85)
5-HIAA	18.2	(15.1 – 21.3)	109.2	(91.0 – 127.4)
HVA	9.1	(7.1 – 11.0)	86.2	(71.4 – 101.0)

The current catecholamine aqueous standard values used are:-

	Noradrenaline	Adrenaline	Dopamine
S1	222	68	422
S2	444	136	844
S3	888	272	1688

Units in nmol/24hr for catecholamines and mmol/24hr for metabolites.



UK NEQAS for Urinary Catecholamines

Laboratory : 1080

Distribution : 38

Date : 4-Nov-96

Page 1 of 6

Distribution Summary

LATE Results

This distribution contained two spiked specimens, and a pooled urine from a female patient with elevated catecholamines and undergoing further investigations.
The urine volume has been assumed to be 1.0 L/24h to avoid confusion between those laboratories that report in units/L and those that report in units/24h.

	Specimen	Pool	Result	Target	Bias	BIS	MRVIS	MRBIS	SDBIS	Median MRVIS
VMA [HMMA] (umol/24h)	127	125	113.0	112	+1.3	+13	197	166	168	130
	128	128	30.0	28.7	+4.5	+45				
	129	129	18.0	14.3	+26.0	+260				
HVA (umol/24h)	127	125	70.0	52.4	+33.6	+336	219	219	127	130
	128	128	40.0	10.7	+273.6	+400				
	129	129	30.0	18.1	+65.4	+400				
Noradrenaline (umol/24h)	127	125	2.690	3.12	-13.9	-139	182	27	203	108
	128	128	1.441	1.89	-23.6	-236				
	129	129	0.261	0.293	-10.9	-109				
Adrenaline (umol/24h)	127	125	0.278	0.390	-28.7	-287	297	-164	257	143
	128	128	0.387	0.551	-29.8	-298				
	129	129	0.763	1.14	-32.8	-328				
Dopamine (umol/24h)	127	125	5.780	5.98	-3.4	-34	117	-117	84	107
	128	128	0.490	0.535	-8.3	-83				
	129	129	0.918	1.12	-18.1	-180				



UK NEQAS for Urinary Catecholamines

Laboratory : 1080

Distribution : 41

Date : 18-Apr-1997

Page 1 of 6

Distribution Summary

This distribution contained a new authentic clinical specimen and two spiked specimens which have been distributed before.
The urine volume has been assumed to be 1.0 L/24h to avoid confusion between those laboratories that report in units/L and those that report in units/24h.

	Specimen	Pool	Result	Target	Bias	BIS	MRVIS	MRBIS	SDBIS	Median MRVIS
VMA [HMMA] (umol/24h)	136	C102	92.0	88.8	+3.6	+36	109	74	112	124
	137	C103	183.0	167	+9.9	+99				
	138	C106	259.0	261						
HVA (umol/24h)	136	C102	35.0	35.7	-1.9	-19	177	171	164	148
	137	C103	59.0	55.6	+6.1	+61				
	138	C106	43.0	43.4	-1.0	-10				
Noradrenaline (umol/24h)	136	C102	1.821	2.15	-15.4	-154	190	-101	201	101
	137	C103	3.890	4.13	-5.8	-58				
	138	C106	2.710	8.43	-67.9	-400				
Adrenaline (umol/24h)	136	C102	0.459	0.541	-15.2	-152	287	-173	240	139
	137	C103	0.702	0.924	-24.0	-240				
	138	C106	0.052	0.096						
Dopamine (umol/24h)	136	C102	4.214	4.75	-11.2	-112	125	-125	77	109
	137	C103	8.138	8.70	-6.5	-65				
	138	C106	2.554	2.98	-14.3	-143				

**UK NEQAS for Urinary Catecholamines**

Laboratory : 1080

Distribution : 45

Date : 12-Dec-1997

Page 1 of 6

Distribution Summary

This distribution contained two diluted clinical specimens, and a spiked normal urine. None of these pools have been distributed before.

The urine volume has been assumed to be 1.0L/24h to avoid confusion between those laboratories that report in units/L and those that report in units/24h. Please note that some analyte concentrations in specimens 148 and 149 were below our lower limits for performance assessment, and therefore no scores have been calculated.

	Specimen	Pool	Result	Target	Bias	BIS	MRVIS	MRBIS	SDBIS	Median MRVIS
VMA [HMMA] (umol/24h)	148	C116	92.0	74.2	+24.0	+240	178	178	138	108
	149	C117	92.0	66.6	+38.2	+382				
	150	C118	119.0	83.1	+43.2	+400				
HVA (umol/24h)	148	C116	14.0				105	80	142	128
	149	C117	14.0	10.4	+35.2	+352				
	150	C118	68.0	53.2	+27.9	+279				
Noradrenaline (umol/24h)	148	C116	4.082	3.57	+14.4	+144	175	37	192	96
	149	C117	2.508	2.19	+14.6	+146				
	150	C118	3.014	2.61	+15.5	+155				
Adrenaline (umol/24h)	148	C116	1.174	1.13	+3.9	+39	111	-2	129	116
	149	C117	0.028							
	150	C118	0.638	0.601	+6.2	+62				
Dopamine (umol/24h)	148	C116	0.682	0.525	+29.8	+298	252	181	206	105
	149	C117	1.011	0.760	+33.0	+330				
	150	C118	3.211	2.44	+31.7	+317				

**UK NEQAS for Urinary Catecholamines**

Laboratory : 1080

Distribution : 47

Date : 24-Apr-1998

Page 1 of 6

Distribution Summary

This distribution contained three spiked acidified urine pools, each pool was prepared from a different base urine. None of these pools have been distributed before.

The urine volume has been assumed to be 1.0 L/24h to avoid confusion between those laboratories that report in units/L and those that report in units/24h.

	Specimen	Pool	Result	Target	Bias	BIS	MRVIS	MRBIS	SDBIS	Median MRVIS
VMA [HMMA] (umol/24h)	154	C122	23.0	18.2	+26.4	+264	228	228	136	103
	155	C123	24.0	13	+84.8	+400				
	156	C124	103.0	92	+12.0	+120				
HVA (umol/24h)	154	C122	42.0	25.8	+62.6	+400	244	226	187	121
	155	C123	56.0	23.4	+138.9	+400				
	156	C124	62.0	45.2	+37.3	+373				
Noradrenaline (umol/24h)	154	C122	0.708	0.687	+3.1	+31	138	81	123	87
	155	C123	1.882	2.29	-17.8	-178				
	156	C124	5.460	5.93	-8.0	-80				
Adrenaline (umol/24h)	154	C122	0.605	0.598	+1.2	+12	63	35	61	110
	155	C123	0.139	0.150	-7.5	-75				
	156	C124	2.206	2.30	-3.9	-39				
Dopamine (umol/24h)	154	C122	1.745	1.48	+18.0	+180	260	250	125	99
	155	C123	2.297	1.98	+15.8	+158				
	156	C124	3.970	4.15	-4.4	-44				

**UK NEQAS for Urinary Catecholamines**

Laboratory : 1080

Distribution : 48

Date : 26-Jun-1998

Page 1 of 6

Distribution Summary

This distribution contained a normal acidified urine pool and a spiked pool from the same base urine. The third specimen was a pooled urine from a 57 year old male patient with a confirmed metastatic carcinoid tumour. This specimen had been diluted slightly to allow us sufficient volume for the distribution.

None of these pools have been distributed before.

The urine volume has been assumed to be 1.0 L/24h to avoid confusion between those laboratories that report in units/L and those that report in units/24h.

	Specimen	Pool	Result	Target	Bias	BIS	MRVIS	MRBIS	SDBIS	Median MRVIS
VMA [HMMA] (umol/24h)	157	C125	35.0	22.9	+52.7	+400	253	253	131	129
	158	C126	23.0	17	+35.2	+352				
	159	C127	199.0	161	+23.6	+236				
HVA (umol/24h)	157	C125	115.0	88.6	+29.8	+298	224	210	170	133
	158	C126	20.0	18	+11.3	+113				
	159	C127	55.0	50.4	+9.0	+90				
Noradrenaline (umol/24h)	157	C125	0.439	0.476	-7.8	-78	122	42	129	86
	158	C126	0.224	0.262	-14.4	-144				
	159	C127	6.380	6.43	-0.8	-8				
Adrenaline (umol/24h)	157	C125	0.086				74	14	84	108
	158	C126	0.043							
	159	C127	1.701	2.03	-16.3	-163				
Dopamine (umol/24h)	157	C125	0.671	0.660	+1.6	+16	214	174	178	105
	158	C126	0.876	0.863	+1.5	+15				
	159	C127	3.020	3.76	-19.6	-196				

**UK NEQAS for Urinary Catecholamines**

Laboratory : 1080

Distribution : 49

Date : 21-Aug-1998

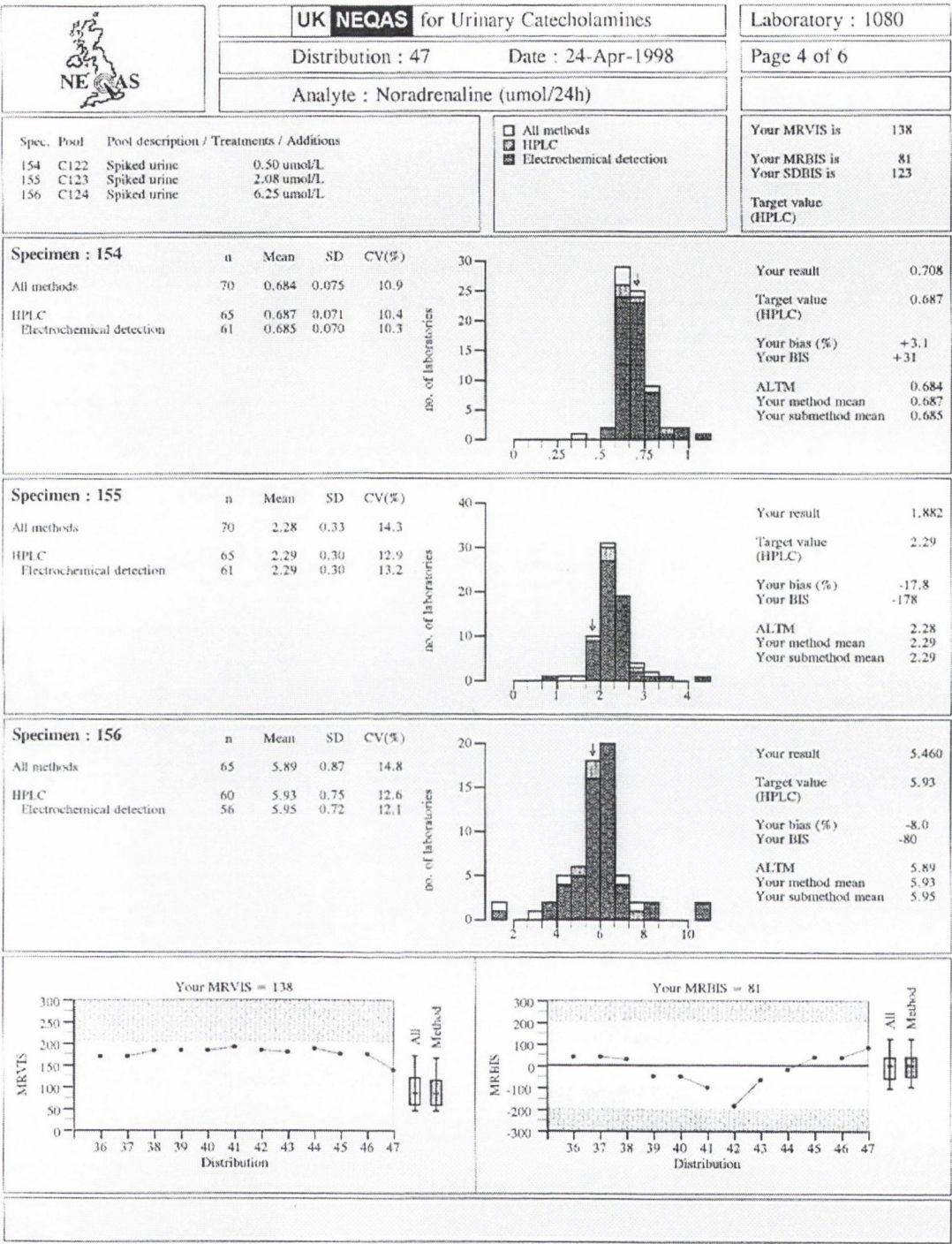
Page 1 of 6

Distribution Summary

This distribution contained three spiked acidified urine pools, each pool was prepared from a different base urine. These three pools have previously been distributed at distribution 47 (April 98).

The urine volume has been assumed to be 1.0 L/24h to avoid confusion between those laboratories that report in units/L and those that report in units/24h.

	Specimen	Pool	Result	Target	Bias	BIS	MRVIS	MRBIS	SDBIS	Median MRVIS
VMA [HMMA] (umol/24h)	160	C122	20.0	18.8	+6.6	+65	271	271	120	124
	161	C123	14.0	12.6	+11.1	+111				
	162	C124	115.0	93.4	+23.1	+231				
HVA (umol/24h)	160	C122	29.0	26.8	+8.4	+84	232	228	146	123
	161	C123	23.0	23.5	-2.1	-21				
	162	C124	53.0	46.9	+12.9	+129				
Noradrenaline (umol/24h)	160	C122	0.580	0.660	-12.1	-121	111	-32	125	84
	161	C123	1.671	2.19	-23.7	-237				
	162	C124	5.800	5.95	-2.6	-26				
Adrenaline (umol/24h)	160	C122	0.479	0.592	-19.1	-191	93	-68	99	112
	161	C123	0.116	0.151	-23.1	-231				
	162	C124	2.190	2.25	-2.8	-28				
Dopamine (umol/24h)	160	C122	1.426	1.45	-1.9	-19	142	95	159	104
	161	C123	1.896	1.93	-1.7	-17				
	162	C124	4.500	4.12	+9.1	+91				



UK NEQAS, Wolfson EQA Laboratory,
PO Box 3809, BIRMINGHAM B15 2UE, UK
Phone (direct) 0121-414 7300; FAX 0121-414 1179

© Copyright The data in UK NEQAS reports are confidential,
and participants should contact the scheme Organiser before
quoting data from the Scheme

Printed at 15:37 on Tuesday 28 April 1998

Variance Index (VI). The mean value of all laboratories using the same method is calculated. The method mean is calculated from these values within +/- 3sd from the mean. The method mean (x_m) is subtracted from the result of an individual laboratory (x) and the percentage variation V from the method mean is calculated.

$$V = \frac{x - x_m}{x_m} \times 100$$

The variance index is calculated from the V by dividing it by the chosen co-efficient of variation (CCV). The lower the VI, the closer the result is to the method mean.

$$VI = \frac{V}{CCV} \times 100$$

- MRVIS = Mean running variance index score – mean of most recent 10 scores for each analyte.
- BIS = Bias index score gives the sign direction of bias rather than a simple VIS
- MRBIS = Mean running bias index score
- SDBIS = Standard deviation of the 10 most recent BISs.
- Median MRVIS = Shows the values from laboratories in our group.

Note:

With regard to distribution 41, dated 18 April 1997, the target value for noradrenaline, No C106, was 8.43 and our value given was 2.71, whereas the real value which we had was 7.88. This error was a computer calculation error from the raw data adjustment to the volume of the sample. This was telephoned through later but the reports were not adjustable and therefore, the BIS on that date was off the scale at – 400. This would have continued to effect our next ten MRVIS and MRBIS values.

Reference values for catecholamine secretion related to urine creatinine in adults.

Values of creatinine, noradrenaline, adrenaline and dopamine from 50 hypertensive adult patients, who were being screened for phaeochromocytoma using 24hr urine collections, were used to assign a practical higher reference limit in relation to creatinine excretion. To test the data for Gaussian distribution, a normal probability plot of the data versus N scores was constructed using Datadesk software, and a regression line added. A straight line for the data implies normal distribution. The data line for dopamine indicates a normal distribution and a histogram of dopamine values is also shown. The upper limiting value was taken to be the mean + 2sd. This is 0.21 mmol/creatinine for dopamine. There was one outlying value in the noradrenaline, adrenaline and dopamine columns and these were eliminated from the calculations. These values were noradrenaline 0.06, adrenaline 0.018 and dopamine 0.58 in mmol/mol creatinine. The outliers are eliminated using the Dixon range statistic¹. A possible outlier is rejected if the distance between it and its nearest neighbour is greater than 1/3 of the range. The regression lines and histograms for noradrenaline and adrenaline show that these are not normally distributed. Neither square root nor log transformation of the data for noradrenaline and adrenaline results in a normal distribution. The data for dopamine are Gaussian and the mean + 2sd cut-off value is <0.21 mmol/mol creatinine. Previously, Ross et al found that the values in 106 patients for 24 hr urinary dopamine were normally distributed and that the distribution of values for noradrenaline and adrenaline were skewed and approximated to a normal distribution following log transformation². They draw attention to the incorrect presumption of a Gaussian distribution for noradrenaline and adrenaline excretion in many of the published reference ranges. When determining the upper reference limit by age in children, a line was drawn at the top of the data points having eliminated outliers³. For convenience, we used the same method for adult adrenaline and noradrenaline, and the upper reference limits in adults are 0.01 and 0.04 mmol/mol creatinine respectively. Catecholamine values are higher in hypertensive patients than in normotensives and the former group is used for reference to avoid the consequences of investigating many more false positive values⁴⁻⁶.

The ages ranged from 20 to 93 with a median value of 54 years. There were 11 females and 39 males.

References:

1. Solberg HE. Approved recommendation (1987) on the theory of reference values. Part 5. Statistical treatment of collected reference values. Determination of reference limits. Clin Chim Acta 1987;170:S13-S32.
2. Ross GA, Newbound EC, Thomas J, Bouloux PMG, Besser GM, Perrett D, Grossman A. Plasma and 24h-urinary catecholamine concentrations in normal and patient populations. Ann Clin Biochem 1993;30:38-44.

3. Fitzgibbon M, Fitzgerald RJ, Tormey WP, O'Meara A, Kenny D. Reference values for urinary HMMA, HVA, noradrenaline, adrenaline and dopamine excretion in children using random urine samples and HPLC with electrochemical detection. *Ann Clin Biochem* 1992;29:400-404.
4. Jones DH, Hamilton CA, Reid JL. Choice of control groups in the appraisal of sympathetic nervous activity in essential hypertension. *Clin Sci* 1979;57:339-44.
5. Miura Y, Kobayashi K, Sakuma H, Tomioka H, Adachi M, Yoshinaga K. Plasma noradrenaline concentrations and haemodynamics in the early stage of essential hypertension. *Clin Sci Mol Med* 1978;suppl 4:69s-71s.
6. Kobayashi K, Kolloch R, Dequattro V, Miano L. Increased plasma and urinary normetanephrine in young patients with primary hypertension. *Clin Sci* 1979;57 suppl 15:173s-176s.

Results and case details in pheochromocytoma and paraganglioma

In this chapter, the case details on patients with paragangliomas, pheochromocytoma alone, pheochromocytoma and neurofibromatosis, pheochromocytoma and von Hippel Lindau are set out as are those cases where multiple endocrine neoplasia (MEN) type II and type I have been evaluated. These are the cases in which the department has had direct or indirect involvement from 1988 to 1999 inclusive. Histological confirmation of the tumour is taken as the gold standard for inclusion.

The number of patients diagnosed has been relatively high by international standards. For example, at the Middlesex and University College Hospital in London between 1952 and 1982, 54 patients with pheochromocytoma were diagnosed¹. The reported incidents at the Mayo Clinic in Rochester, Minnesota is approximately 8 per million and 1-2 cases per million per year is the figure claimed from cases in the Netherlands². In Sweden³, where all cases are registered on a tumour registry, the annual incidence is 2 per million. In the Republic of Ireland, there may be about 6 cases per year for a population of approximately 3 million people.

Case details - Sporadic pheochromocytomas 26. (One patient subsequently developed a Carotid Body Tumour, 13 years after adrenal surgery)
Von Hippel Lindau and pheochromocytoma 3
Neurofibromatosis and pheochromocytoma 3
Malignant paraganglioma – 1
Ganglioneuroblastoma in an adult. *1

There were no confirmed cases of Multiple Endocrine Neoplasia (MEN). However, one patient had a pheochromocytoma in 1980 and a carotid body paraganglioma in 1993 and also has adenomatous polyposis of the colon. This raises the possibility of MEN 2b.

Two cases occurred during pregnancy. Both were delivered by caesarian section and later had the pheochromocytomas removed. Both presented with hypertension. Patient 1 had paroxysms of sweating, palpitations, flushing, headache and blurred vision and pheochromocytoma was suspected at the first antenatal visit at 30 weeks. Blood pressure rose to 230/120 during paroxysms. In 1999, the patient was normotensive and did not require drug therapy.

Patient 2, was diagnosed at 28 weeks of pregnancy and had a Caesarian section at 34 weeks because of small-for-dates and abnormalities on foetal cardiac monitoring. Nine years earlier, she had a pheochromocytoma removed following a miscarriage and had hypertension.

- neuroblastoma and ganglioneuroblastoma may give rise to paroxysmal hypertension

Anatomical sites of tumours

Adrenal gland. Right 15;
Left 8
Bilateral 6

Posterior thorax 1

Left para-aortic area at the bifurcation at L₂ level. 1

At the Coeliac Plexus on right of midline in upper abdomen.1

Malignant abdominal paraganglioma – primary site unknown.1

Ganglioneuroblastoma.1

There were 20 males and 14 females. The median age was 34 years. The lower age quartile was up to 25 years and the upper age quartile was from 51 years. The youngest and oldest patients were 8 and 76 years respectively. The age cited in patients with bilateral adrenal tumours was taken as that at the time of first diagnosis.

Arterial hypertension - Sustained in 26 with superimposed paroxysms in 7 of these
- Paroxysmal surges in blood pressure on a normotensive background in 5
- Normotensive 1 patient.
- No blood pressure data in 2 patients.

Symptoms and signs

The following symptoms and signs were recorded in the case records of 31 patients. The two patients where the clinical symptoms remain unknown are SW a 36 year old lady with a left adrenal phaeochromocytoma in 1989 and F O'D a 65 year old farmer with abdominal metastatic paraganglioma.

Headache 15

Palpitations 14

Sweating 13

Tachycardia 7 (two cases with atrial tachycardia and the remaining 5 had sinus tachycardia)

Abdominal pain 6

Flushing 6

Lethargy 4

Anxiety 4

dyspnoea 4

nausea 3

cold 3

blurred vision 2

glycosuria 2

weakness 1

Polydipsia 1

dizziness 1
vomiting 1
skin mottling 1
paraesthesia 1
bony chest pain 1
chest tightness 1
weight loss 1
neck pain 1
No symptoms 1

Imaging

The imaging modalities available to clinicians have improved over time. Thus CT scans were not available until the mid to late 1980s, MRI until the mid 90s. Ultrasound was widely available except for the woman who had a tumour excised in London in 1954.

Abdominal ultrasound was reported in 12 patients and all except one were positive. The negative scan was performed two years before the CT which found an unexpected mass in a 34 year old patient admitted for the investigation of a recurrence in haemoptysis and who had hypertension for three years. All 4 MRI scans were positive. There was one false negative CT scan among the 22 patients who were scanned. One of these CT examinations was done as a follow-up and was negative as were the urinary catecholamines. There was also one false negative among the 12 patients who had an ^{123}I MIBG scan.

Pre-operative antihypertensive drug therapy

Data was available in 18 patients.

Labetalol was used in 13 patients.
Phenoxybenzamine in 13
Propranolol in 6
Amlodipine in 2
Nifedipine in 3
Doxazosin in 2
Atenolol in 1
Hydralazine 1
Alpha-methyldopa 1
Enalapril in 1

Intraoperatively at Beaumont Hospital, esmolol, sodium nitroprusside and phentolamine were used routinely to control arterial blood pressure.

Biochemical diagnosis

The sensitivities for phaeochromocytoma diagnosis from 1989 to December 1999 are

For noradrenaline	sensitivity = 87.7% from 57 samples from 30 cases. (79%)
adrenaline	sensitivity = 48.1 % from 54 samples from 30 cases (51.5%)
dopamine	sensitivity = 20% from 55 samples from 30 cases (12%)
HMMA	sensitivity = 74.1% from 54 samples from 27 cases. (60%)
HVA	sensitivity = 28% from 25 samples from 15 cases

The published sensitivities for this department for 1989 to 1994⁴ are shown in brackets.

Urine metanephrines were measured as part of the contemporaneous diagnostic screen in 3 patients. The values were raised in all three. Two also had increased urine catecholamines and the other patient, only HMMA had been measured and this was normal.

Plasma catecholamines were measured in 3 patients all of whom had raised urine catecholamines in contemporaneous urine collections. The plasma levels reflected the urine values in each case and did not add to the diagnosis.

If test results are considered in batches in line with the investigation protocol published in 1995⁴, then the value of biochemical screening can be made to appear more sensitive. By taking test results in batches for each patient, and accepting the proposition that a single raised value within a patient data set confers a positive result for an analyte in an individual patient, sensitivities for the diagnosis of phaeochromocytoma can be recalculated. Thus batch sensitivities are 100% for noradrenaline in 28 cases, 64.3% for adrenaline in 28 cases, 28.6% for dopamine in 28 cases, 88% for HMMA in 25 cases, 25% for HVA in 16 cases.

A retrospective analysis for metanephrines in stored pre-operative samples from 10 patients with phaeochromocytoma from 1996 was undertaken. The maximum duration of freezing at -20° C was 4 years. Individual results are shown graphically below.

For these cases, the sensitivities were

For total normetanephrine	sensitivity = 93.3% from 15 samples from 10 cases.
total metanephrine	sensitivity = 33.3% from 15 samples from 10 patients.

Using all the available data including these 10 patients and the three patients noted above the

Total metanephrine sensitivity = 94.4% from 18 samples from 13 patients.

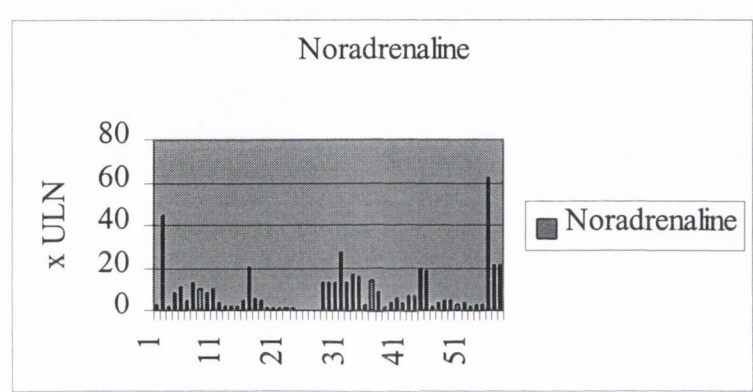
Ratios of noradrenaline and adrenaline to dopamine in phaeochromocytoma.

The ratio varied from 0.02:1 to 22.48:1 for noradrenaline and 0.05:1 to 19.2:1 for adrenaline.

To allow every patient result to be shown together, the values were related to the upper limit of normal whether these were reported in nmol/24 hours or in mmol/mol creatinine or in nmol/L for the catecholamines and metanephrines or in $\mu\text{mol}/24\text{ hr}$ for HMMA and HVA. Thus the relative increment in spot or timed urine samples can be compared.

The 57 noradrenaline values for the patients preoperative urine collections shown as multiples of the upper limit of normal (x ULN) are shown in figure 1 below.

Figure 1



All 54 adrenaline pre-operative values are shown as multiples of the upper limit of normal (x ULN) in figure 2 below. The apparent spaces on the abscissa are due to adrenaline values within the reference interval.

Figure 2.

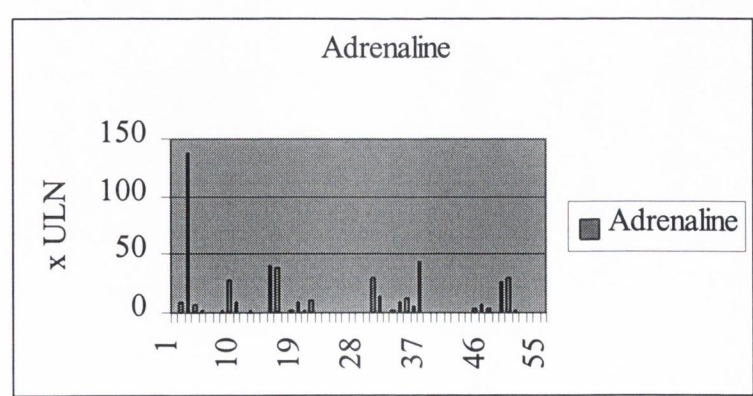
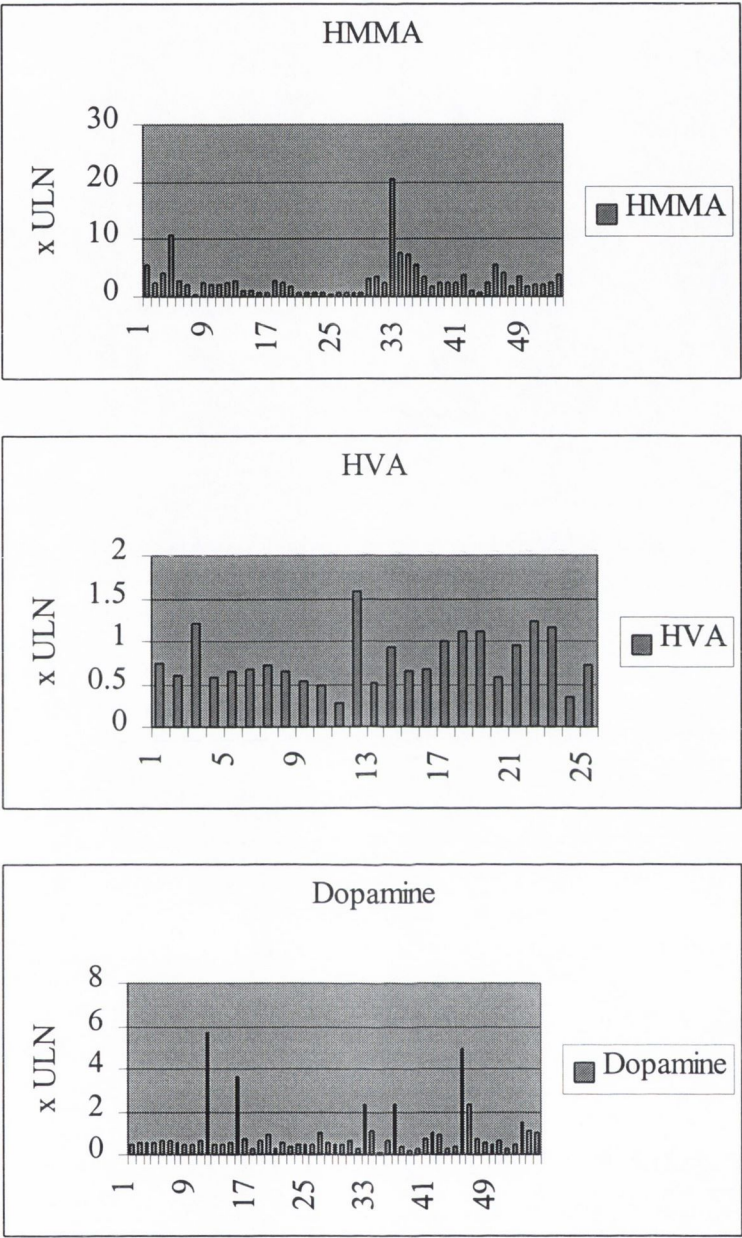
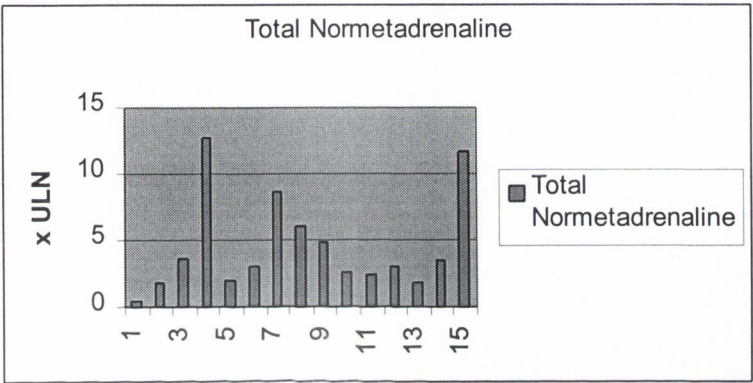


Figure 3



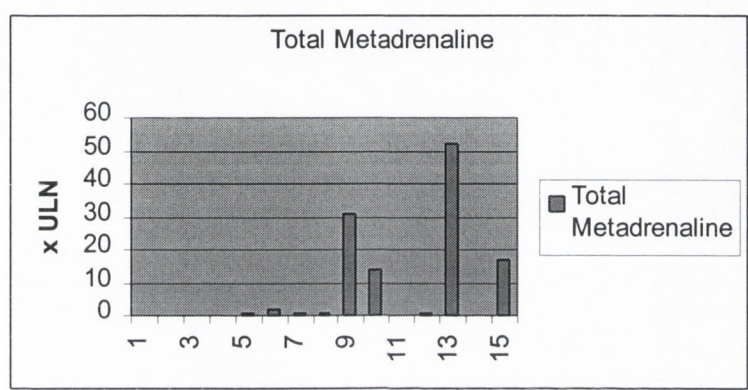
All 54 HMMA values, all 25 HVA values and all 55 dopamine values from pre-operative urines are shown in figure 3 above. Values on the abscissa or below x1 ULN are within the reference range

Figure 4



Graphical illustration of pre-operative normetanephrine values as multiples of the upper limit of normal (x ULN) is shown in figure 4 on the previous page. There were 15 data points from 10 patients with phaeochromocytoma. The upper reference limit is taken as 5100 nmols as per Graham et al⁵. Two of the patients were children and the appropriate reference value for age was taken from Tietz Textbook of Clinical Chemistry. Using a similar method to here, the upper reference limit calculated from data in drug treated hypertensives was 4000 nmol/day. This figure would not alter the sensitivity data reported here.

Figure 5



Graphical illustration of 15 data points of pre-operative total metanephrine values in 15 patients with phaeochromocytoma is shown in figure 5 above. Data falling on the abscissa are within the reference range. The upper reference limit was 2000 nmols per day taken from Graham et al⁵. Two of the patients were children and the appropriate reference value for age was taken from Tietz' Textbook of Clinical Chemistry. Hope Hospital in Salford, England use a similar method to here and found the 2000 nmol/day was the upper limit in drug treated essential hypertensive patients.

Figure 6

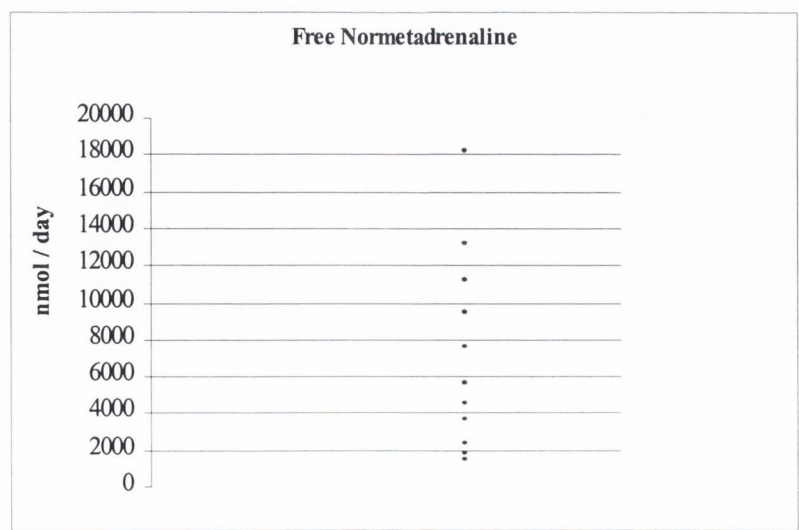
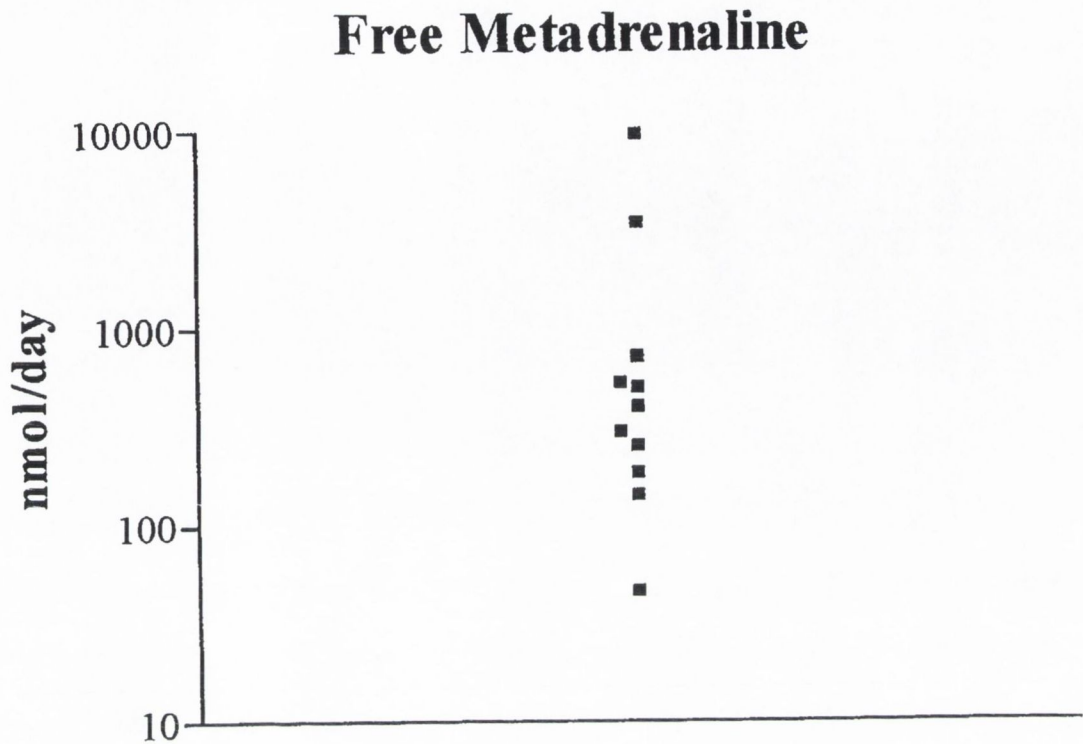


Figure 7



Free normetanephrine and free metanephrine values are shown in figures 6 and 7 above for 11 data points from 8 patients with phaeochromocytoma. A log scale is used for the ordinate in figure 7 only. There are no international reference values published. The data are presented in nmols / day.

Familial syndrome biochemical screens

Thirteen patients with sporadic phaeochromocytoma were screened for MEN 2

- Calcitonin in 9
- Parathyroid hormone in 4
- Serum calcium in 9

Details of the investigations are listed below: Each refers to a separate patient.

1. Calcitonin, PTH, cortisol, calcium, potassium (patient also has primary hyperaldosteronism).
2. Calcitonin, PTH.
3. Calcitonin, PTH, calcium
4. Calcitonin, gastrin.
5. Calcitonin, calcium
6. Calcitonin, calcium
7. Calcitonin, calcium
8. Calcitonin, prolactin and calcium
9. Calcium

10. Calcium
11. Calcium
12. PTH.
13. Calcitonin and gene mutation screen.

In summary, one patient had a screen for MEN 1 and 2. with gastrin, and calcitonin measured. Another had prolactin as well as calcitonin and serum calcium. A further patient had PTH, cortisol and calcium as well as calcitonin. Calcitonin and either PTH and/or calcium were measured in 7 patients.

The 34 year old patient with a thoracic tumour had a post-operative raised calcium of 2.75 mmol/L but there were bony secondaries and the alkaline phosphatase was elevated. So bony metastases could not be excluded as the most likely cause. The family has not been systematically screened for occult pheochromocytoma, but Von Hippel Lindau is unlikely. Two requests for PTH entered into the patients clinical notes were not executed.

A detailed family history was taken in a further patient to exclude MEN but no hormone markers were measured. A 10 year old male was tested for gene mutations at 618/620/634 and the genes were normal.

Detailed histories were taken in the three patients with neurofibromatosis and pheochromocytoma - one had PTH and calcitonin measured and another had a normal calcium. One of the index neurofibromatosis patients has a family history atypical for neurofibromatosis type 1 and there is a possibility that the disorder may be a variant of Li-Fraumeni syndrome. (a syndrome of early breast cancer associated with soft tissue sarcomas and other tumours.)

Similarly detailed family histories were taken in the patients with Von Hippel Lindau and pheochromocytoma. Two patients were from the same family One patient with VHL had a normal calcitonin and a positive missense mutation 683 C>T. Two siblings have the identical missense mutation but currently have normal urine catecholamine excretion and CT brain scans. The mother had bilateral pheochromocytomas and a retinal tumour.

The third patient (JC) with VHL is from a very large family with a demonstrated gene deletion and is the only family member to have had a pheochromocytoma.

Outcomes

There were six deaths from 34 cases (17.64%) due directly or indirectly to the paraganglioma. Of the 29 patients with adrenal pheochromocytoma, 3 (10.3 %) died. The patient with ganglioneuroblastoma had the diagnosis specified following autopsy.

Follow-up of survivors

The longest surviving period post-surgery is 45 years. Of those 27 patients with adrenal tumours who survived the peri-operative period, no metastatic recurrence has been recorded.

By November 1999,

Five patients have been followed for ten years or more, Eleven patients from 5 to 9 years and 11 months. Eleven patients up to 4 years and 11 months. Two patients are within the immediate post-operative period in December 1999.

In cases with sequential second tumours, these survival figures list time periods from the original tumour excision. Two patients had their initial tumours 19 years and 6 months and 17 years and 5 months earlier. In those patients with bilateral phaeochromocytoma, three were discovered contemporaneously. For the three other bilateral tumour patients, there was an interval of 2, 2, and 9 years between their excisions. Three patients with Von Hippel Lindau disease had bilateral phaeochromocytoma.

No cases of MEN were identified as all biochemical values in the eight cases where measured were within the reference range.

Arterial hypertension was eliminated by surgical removal of the tumour in 21 of 30 cases (70%) where surgery was performed. In the other cases, two patients died before surgery was possible, and in one metastatic paraganglioma made surgery inappropriate and in the patient with ganglioneuroblastoma, death occurred from septicaemia before a tissue diagnosis was made. Hypertension persisted in 4 (13.3%).

Hyperaldosteronism

One patient whose blood pressure remained elevated following surgical excision of the phaeochromocytoma has primary hyperaldosteronism with an aldosterone/plasma renin activity ratio of 1179.6 (ref 28 – 924). The serum aldosterone was 696 pmol/L (140 – 1400) and the plasma renin activity was 0.59 ng/ml/hr (0.5 – 6.8). The plasma K was normal at 4.3 mmol/L. The patient with the malignant phaeochromocytoma in the posterior thorax also had a raised aldosterone pre-operatively but the plasma renin activity was not done. Further investigation was not pursued because of the patient's clinical condition. None of the other patients with phaeochromocytoma were specifically investigated for coexistent hyperaldosteronism.

Changes in catecholamine, HMMA and HVA secretion following surgery:

Post-surgery values were available from twelve patients. However, in one case the tumour sited in the posterior thorax had invaded the spine at T8 and therefore could only be partially removed. The catecholamines in this patient later acted as a tumour marker following the course of the disease. The data are shown separately in Figure 1 below. In another case, the original tumour had been removed more than 40 years ago and no historic pre-operative data is available.

Where there were more than one 24 hour collection, the pre and post operative means were calculated and the changes shown. Pre and post-operative data is available for catecholamines from another ten cases. Data for HMMA and HVA are available from 9 and 4 cases respectively. The excretion of all analytes fell following surgery.

The mean change in urinary excretion was

urinary noradrenaline	- 94.5%,	n = 10
urinary adrenaline	- 36.7%	n = 10
urinary dopamine	- 5.8%	n = 10
urinary HMMA	- 73.7%	n = 9
urinary HVA	- 14.2%	n = 4

Details of the values for the pre-operative and post-operative collections are shown in table 1 which is inserted immediately following figure 1 on page 108.

In the above mentioned case with spinal invasion, noradrenaline was used as an indicator tumour marker following the surgical excision of September 4 1994. Surgery was performed in early September 1994 and the patient died from metastatic disease in 1996 despite irradiation therapy which included labelled MIBG. The data is shown in figure 1, page 108.

No patient in this series had evidence of renal impairment as evidenced by an increased plasma creatinine.

Figure 1 (overleaf)

Legend: - The points in time shown on the x-axis represent the dates of 24 hour urinary noradrenaline collections as follows 1. August 1994 pre-operative value, 2. Two weeks after surgery, 3. Three weeks post surgery, 4. June 1995, 5. July 1995 following radiotherapy with labelled MIBG, 6. July 1995 and 7. August 1995.

urinary noradrenaline as a tumour marker

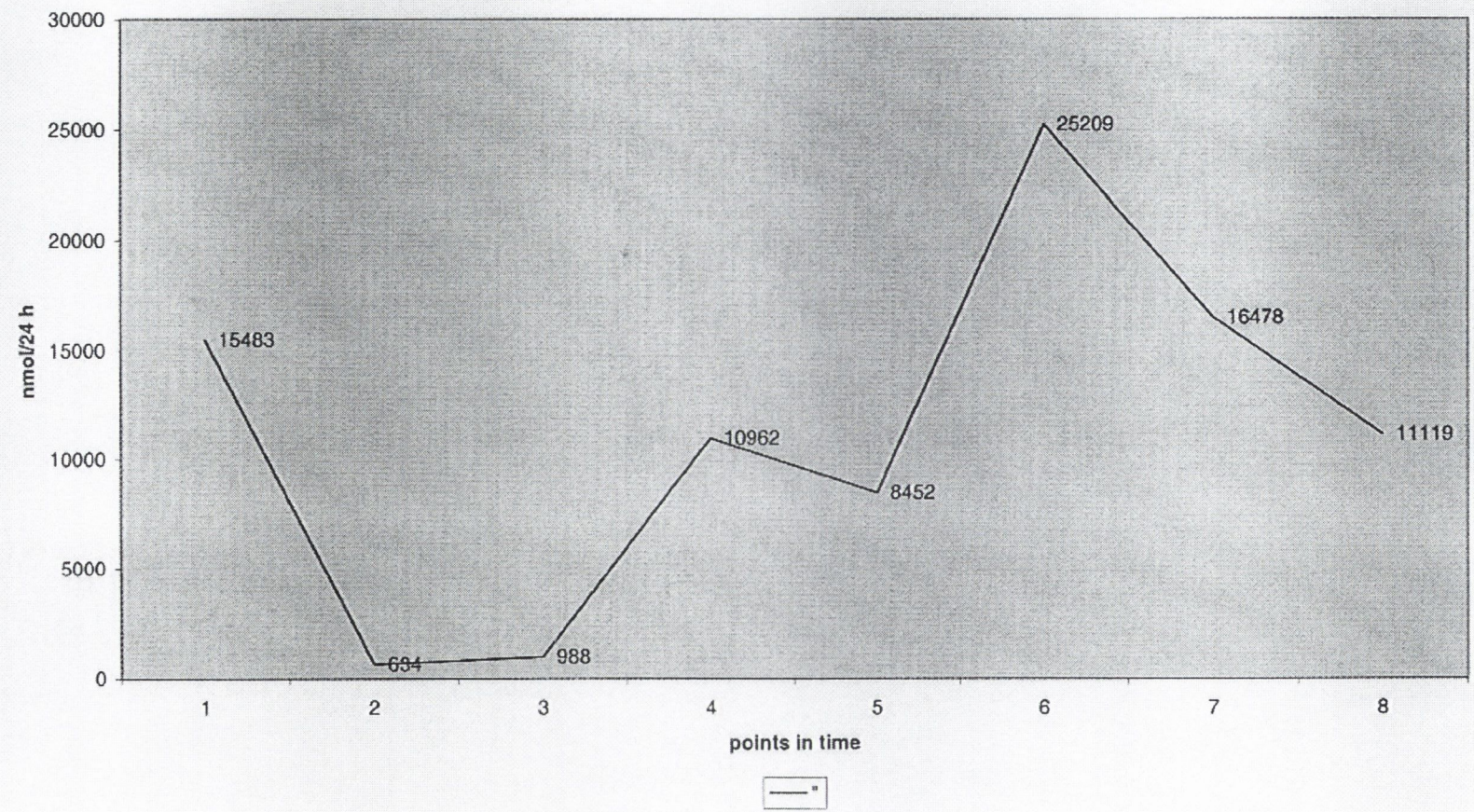


Table 1		Chapter 6							
Noradrenaline				Adrenaline					
Pre-Surgery	Post-Surgery	Difference	% Change		Pre-Surgery	Post-Surgery	Difference	% Change	
8851	356.75	-8494.25	-96.0%		336	92	-244	-72.6%	
n = 2	n = 3				n = 1	n = 3			
8,239	439	-7800.00	-94.7%		129	222.75	Plus 93.75	Plus 72.67%	
n = 2	n = 4				n = 2	n = 4			
4723	210	-4513	-95.50%		274.5	75	-199.5	-72.70%	
n = 2	n = 1				n = 2	n = 1			
9,600	425	-9175	-95.6%		296	32	-264	-89.2%	
n = 1	n = 1				n = 1	n = 1			
2.15	0.05	-2.1	-97.7%		0.03	.018.	-0.012	-40.0%	
n=3	n = 3				n = 3	n = 3			
12,375	1,024	-11,351	-91.7%		2,976	184	-2792	-93.8%	
n = 1	n = 1				n = 1	n = 1			
2774.5	281	-2493.5	-89.9%		1,138	269.5	-868.5	-76.3%	
n=2	n = 2				n = 2	n = 2			
2,286	297	-1989	-87.0%		26	36.5	Plus 10.5	Plus 40.4%	
n = 2	n = 2				n = 2	n = 2			
10.57	0.04	-10.53	-100%		0.008	0.009	Plus .001	Plus 12.5%	
n = 1	n=1				n = 1	n = 1			
19103	545.5	-18557.5	-97.10%		109	56	-53	-48.60%	
n = 2	n = 2				n = 2	n = 2			
		Mean	-94.5%				Mean	-36.8%	
Dopamine					HMMA				
Pre-Surgery	Post-Surgery	Difference	% Change		Pre-Surgery	Post-Surgery	Difference	% Change	
1,718	2292.5	Plus 574.5	Plus 33.4%		70	42.75	-32.75	-46.8%	
n = 1	n = 3				n = 3	n = 1			
2,167	2,434	Plus 267	Plus 12.3%		99	51.4	-47.6	-48%	
n = 2	n = 4				n = 3	n = 3			
1740	1609	-131	-7.50%		92	21.3	-70.7	-76.80%	
n = 2	n = 1				n = 2	n = 1			
1,863	1629	-234	-12.5%		128.1	18.5	-109.6	-85.5%	
n = 1	n = 1				n = 1	n = 1			
0.36	0.27	-0.09	-25.0%		23.4	3.63	-19.77	-84.5%	
n = 3	n = 3				n = 3	n = 3			

2,017	3,564	-1547	Plus 76.69%	249	16	-233	-93.6%
n = 1	n = 1			n = 1	n = 1		
12,059	2719	-9340	-77.4%	44.5	15.5	-29	-65.2%
n = 2	n = 2			n = 2	n = 2		
1617	1810	Plus 193	Plus 11.9%				
n = 2	n = 2						
1.15	0.32	Plus 0.83	-27.80%	29.1	4.1	-25	-85.90%
n = 1	n = 1			n = 1	n = 1		
3505	1043	-2462	-70.20%	151	33.65	-117.4	-77.70%
n = 2	n = 2			n = 1	n = 2		
		Mean	-5.8%		Mean	-73.7%	
HVA							
Pre-Surgery	Post-Surgery	Difference	% Change				
5.0	4.9	-0.1	-2%				
n = 3	n = 3						
37.3	32	-5.3	-14.2%				
n = 1	n = 1						
4.1	3.9	-0.2	-4.90%				
n = 1	n = 1						
28	18	-10	-35.70%				
n = 1	n = 2						
		Mean	-14.2%				
Changes in the excretion of catecholamines, HMMA and HVA following tumour excision.							
n = number of samples per patient.							
noradrenaline, adrenaline and dopamine values in nmol/24 hr, HMMA in umol.24 hr.							
Two cases have analyte values per mol creatinine							

Histology

A standard reporting procedure does not operate for solid tumours at present.

No extracapsular extension was specifically reported in 13 cases. There was extra capsular spread was noted in 5 cases including two with metastatic disease.

There were two cases where metachronous tumours were noted in one adrenal gland.

Consistent tumour weights and dimensions are not available to list all tumours.

Tumour weights are available from 10 cases. The mean weight is 111.1 g, the minimum was 14 g and the maximum 400 g.

The specimen diameter only is available in 8 cases with a total of 9 tumours. The mean is 5.2 cm, and the range is from 1.4 to 10 cm.

Two dimensions are available for 21 tumours. The mean value is 23.9 sq cm, the lower quartile is up to 12 and the upper quartile is above 32.5 sq cm.

Three dimensions were listed for 13 tumours from 12 cases. The mean value is 70.5 cm³ and the range is from 10 to 160 cm³. The median value is 75 cm³, the lower quartile is up to 24 and the top quartile is above 120 cm³.

Using Pearson's Correlation Co-efficient, there was no significant correlation between the tumour size in cm³ and the mean noradrenaline secretion for each case $r = 0.11$, 95% confidence interval -0.50 to 0.64, 2 tailed $p = 0.736$ – Figure 2.

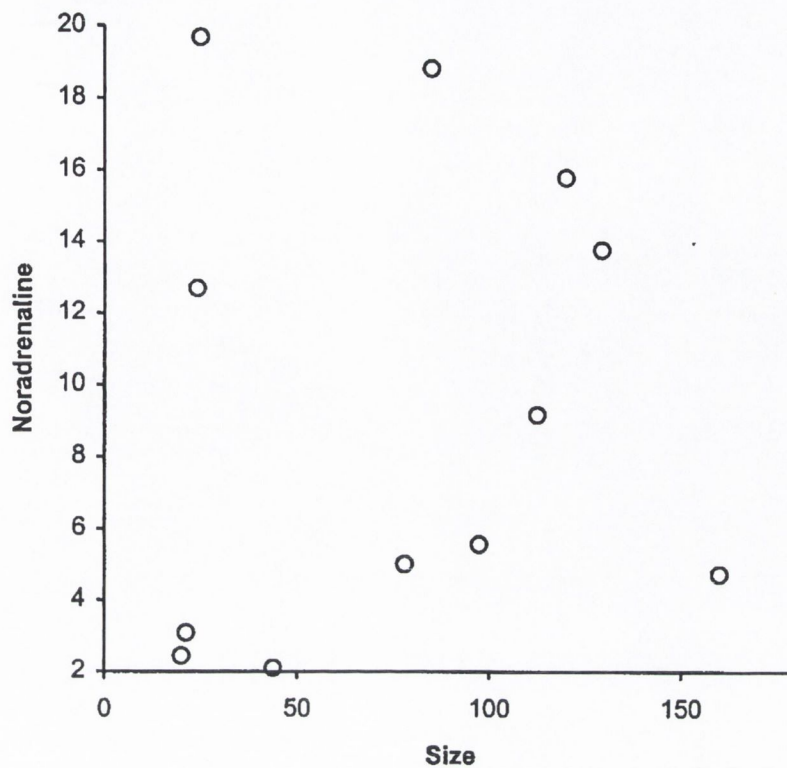


Figure 2.

Noradrenaline in x upper limit of normal and tumour size in cm³.

Gene testing

Gene testing for mutations in codon 618/620/634 of the RET proto-oncogene were negative in one ten year old boy with a sporadic tumour. The RET proto-oncogene encodes a tyrosine kinase receptor. These codons are in the cysteine –rich extracellular domain important for the dimerization of the RET tyrosine kinase receptor. A mutation in codon 634 which converts a cysteine to an arginine accounts for 50% of all MEN 2 mutations.

One family with Von Hippel Lindau disease were found to have a missense mutation 683 C>T in the index patient who had bilateral pheochromocytoma and in two siblings. A second family with VHL has a gene deletion and the patient is the only family member with pheochromocytoma.

One patient with a positive family history of neurofibromatosis has had gene testing by linkage for NF1.

Two recent patients aged 9 and 13 years have had DNA sent for VHL and MEN 2 testing.

Thus by January 2000, DNA had been tested in a total of 7 patients, all recent cases.

Causes of death

1. Lactic acidosis and cerebral oedema shortly after admission with a right adrenal phaeochromocytoma⁶.
2. Adult Respiratory Distress Syndrome (ARDS) and disseminated intravascular coagulopathy with overwhelming infection and the patient died three days following admission to hospital with a left adrenal phaeochromocytoma.
3. Thoracic metastatic paraganglioma with cerebral metastases.
4. Post-operative ARDS with renal, cardiac and gastrointestinal organ failure and septicaemia following surgery for left adrenal phaeochromocytoma.
5. Malignant paraganglioma with widespread intra-abdominal metastases.
6. Cardiac arrest and pneumonia prior to definitive diagnosis, made at postmortem examination in a patient with ganglioneuroblastoma.

Discussion

The anatomical sites and relative distribution of the phaeochromocytoma which is reported here is quite similar to international norms. Phaeochromocytomas are estimated to be present in about 0.3% of patients undergoing investigations for secondary causes of hypertension. A large recent study of 108 phaeochromocytomas in 104 patients over the period 1950 to 1998 was reported from Vanderbilt in Tennessee⁷. There were 66 females and 38 males in contrast to the distribution in this thesis of 20 males and 13 females. The average age at surgery was 42.3 years, about one decade older than in Dublin. Sporadic cases accounted for 84% similar to our figure of 81%. In the Dublin study, there have been no cases of MEN but the US study found cases of von Hippel Lindau and neurofibromatosis with phaeochromocytoma. The malignancy rate of adrenal tumours was 13% with recurrence happening as late as 15 years after resection. The average follow-up was 12.6 years. Over their 26 extra-adrenal tumours, 4 had malignant disease. The overall rate of malignancy was 23% and there was no statistical difference in malignancy rate between the adrenal and extra-adrenal tumours. They conclude that lifelong follow-up is necessary for these patients. Similarly, a recent Italian study of 55 patients with phaeochromocytomas operated upon between 1977 and 1996 also concluded that lifelong follow-up of benign tumours is necessary. They described a recurrence after 183 months in a case thought to be benign⁸.

A Mayo Clinic study of 110 patients with phaeochromocytoma or paraganglioma from 1980 to 1992, 47% had sustained hypertension and classical paroxysmal attacks occurred in 72%. Only 20% required medication for hypertension on discharge. This is the same order of experience as the present Irish study. As in this study, headaches, palpitations and sweating were the most common symptoms. The perioperative mortality was 1% with a morbidity of 16%⁹. In this thesis series, one death was due to

post-operative complications with overwhelming infection leading to multi-organ failure.

In the case noted above with the carotid body second tumour, the patient may have MEN 2b because gastrointestinal manifestations are not commonly recognised in this syndrome¹⁰. Ganglioneuromatosis is a common feature of the disease but it is noted that this patient has adenomatous polyposis. No gene studies have been done to date on this woman.

The apparent improvement in the diagnostic sensitivity of urinary noradrenaline and HMMA, which has occurred since the data published from this department in 1994⁴ reflects the rarity of normal catecholamine secretion in even small volume phaeochromocytomas. In the Mayo Clinic series, above⁹, the combination of urinary metanephrines and HMMA had a sensitivity of 98% in detecting the disease. Interestingly, when the figures in this series are batched for an individual patient, all 28 cases where noradrenaline excretion had been measured had at least one positive result. However, this ignores the real question of the performance of an individual test answering the clinical question.

The changes in the excretion of catecholamines, HMMA and HVA following surgery reflect the intensity of adrenergic drive to the body in tumour victims. Noradrenaline is preferentially secreted and dopamine, its metabolic precursor seems on average little changed after phaeochromocytoma removal. However, from the 10 patients where data on the effects of tumour removal is available, the range of values from -77.4% to +76.7% suggests that firm conclusions cannot be drawn regarding the effects of tumour removal on dopamine excretion in individual cases. Renal genesis of urinary dopamine is a major factor as urinary dopamine excretion is largely accounted for by the uptake and decarboxylation of circulating endogenous dihydroxyphenylalanine (DOPA) by the renal proximal tubular cells¹¹. The low sensitivity of urinary dopamine in phaeochromocytoma diagnosis is consistent with the wide variation in differences between pre- and post-operative values. It also suggests that phaeochromocytoma does not commonly produce a large volume of DOPA. The 94.5% average decrement in urinary noradrenaline output post-surgery when compared with the pre-operative levels is similar to the drop in total urinary metanephrine levels following tumour excision reported in a small series of five cases¹².

The effects of carbidopa in greatly boosting urine dopamine is often experienced in practice. and the effects of paracetamol on the adrenaline chromatography peak make a drug history necessary before interpretation of an increased dopamine or adrenaline in particular.

Noradrenaline and dopamine correlate positively with creatinine clearance but at clearances greater than 40 ml/min, the outputs are similar to those in healthy volunteers. Adrenaline does not correlate with creatinine clearance but excretion is lower in renal failure¹³.

The normal noradrenaline:dopamine and adrenaline:dopamine ratios are 1:5 to 1:10 and 1:10 to 1:50 respectively. With phaeochromocytomas, the ratios are usually

between 1:1 and 1:3 in the published literature^{14,15}. The values in this study overlap with the normal to such an extent that ratios have no role in diagnosis.

Data on metanephrines from 13 patients indicate that urinary normetanephrine may be the best initial test. Hope Hospital in Salford, England using similar laboratory methodology now use urinary metanephrines as the initial screen in phaeochromocytoma and follow-up with free catecholamines where the results are equivocal (Paul Reid, personal communication).

Cardiotoxicity

Noradrenaline is directly toxic to myocardial cells. It acts by cellular calcium overload and the induction of apoptosis¹⁶. Noradrenaline induced apoptosis can be prevented by non-selective beta adrenergic blockade or by combined beta and alpha blockade¹⁶. In heart failure patients, a plasma noradrenaline greater than 800 pg/ml (4.7 nmol/L) indicates a one-year survival of less than 40% unless treated. Thus as well as the effects of hypertension and lactic acidosis, catecholamines also directly affect the heart muscle. Cardiomyopathy due to phaeochromocytoma has been described and is reversible on removal of the tumour¹⁷.

The drugs used to control the pre-operative hypertension reflect the realization that adrenergic blockade is the optimum procedure. Thus the non-selective beta blocker propranolol, the combined alpha and beta blocker labetalol, the cardioselective beta blocker atenolol, the alpha blocker phenoxybenzamine are all used in this condition. Alpha-methyldopa is commonly used in pregnancy hypertension, thus was used to treat the hypertension in one of the pregnant patients. The class 2, calcium antagonists amlodipine and nifedipine were also used but adrenergic blockers were introduced in those patients when the diagnosis of phaeochromocytoma was made.

Histology

It is clear that a standard operating procedure should be used in reporting the weight and dimensions of solid tumours where possible. The smallest dimension nodule which was 1.4 cm in diameter was the tumour that produced the large number of false negative catecholamine results.

The poor correlation between the size of the tumours and the urinary noradrenaline output mirrors the finding of Mihai et al who reported that no correlation was found between noradrenaline output and the number or percentage of noradrenaline-type granules in phaeochromocytomas. They concluded that the lack of correlation between hormone output and granularity suggests that other factors determine the levels of catecholamine secretion by these tumours¹⁸. A Chinese study involving 72 phaeochromocytomas admitted between 1960 and 1995 confirmed no relationship between tumour size and hormone levels¹⁹. However there is evidence that tumour size is a determinant of catecholamine metabolite production but not of the catecholamines themselves^{20,21}.

Aldosteronism

Coexistent phaeochromocytoma and primary aldosteronism is rare enough to warrant the 1998 publication of a case report of a patient with left adrenocortical adenoma causing aldosteronism and a right adrenal phaeochromocytoma²². However, a literature review in 1976 found that an elevated plasma renin activity had been found in more than 70% of patients with phaeochromocytoma, where renin had been measured²³. The dietary salt intake was not controlled in most of these patients. Secondary hyper-reninaemia and secondary hyperaldosteronism from persistent renal arterial vasoconstriction due to direct pressure of a local phaeochromocytoma or from persistent noradrenaline mediated vasoconstriction have been described²⁴. Whether persistent catecholamine vasospasm leads to fibromuscular dysplasia remains unresolved. Circulating catecholamines may cause direct effects on the kidney by stimulation of the juxtaglomerular cells, vasoconstriction of the renal afferent and cortical arterioles, enhancement of sodium reabsorption in the proximal tubular cells and blood volume contraction. Thus it is unsurprising that most patients with phaeochromocytoma have hyperreninaemia.

Pregnancy

There are many case reports in the literature of the rare occurrence of phaeochromocytoma presenting during pregnancy. It may easily be missed as it may mimic some of the features of some of the common hypertension in pregnancy. The tumour may be localised by ultrasound or by MRI and alpha blockers are used to control the blood pressure²⁵. It is unusual that two of the cases in this thesis series were found during pregnancy. Both are well more than 8 years after surgery.

Imaging

There is specific active uptake via the cell membranes and neurosecretory granules in the cytoplasm of ¹³¹I-MIBG in tumours derived from the neural crest resulting in high tumour/non-tumour ratios. In non-adrenergic tissues, uptake is via passive diffusion only. More than 90% of phaeochromocytomas and neuroblastomas, around 35% of medullary thyroid carcinomas, and 70% of carcinoid tumours concentrate MIBG²⁶.

¹³¹I MIBG has sensitivity shortcomings largely because the uptake significantly correlates with the size of the tumour²⁷. Interestingly, a recent report claims that positron emission tomography (PET) with 2-fluorine-18 fluoro-2-deoxy-D-glucose PET is especially useful in defining the distribution of those phaeochromocytomas that fail to concentrate MIBG²⁸. These scanners are not available for clinical use in this environment at present and were not used in any of our patients in any other institution.

¹³¹I MIBG was used therapeutically in our patient with bony metastases but the response was poor. A combination of chemotherapy and ¹³¹I MIBG has been reported to have additive effects in reducing the presence and function of malignant phaeochromocytoma²⁹. False positive ¹²³I-MIBG scintigraphy has rarely been reported³⁰. Pooled results in 117 patients found an overall response rate of 56% and there was subjective improvement and decrease in blood pressure in more than 60%³¹.

In neuroblastoma, therapeutic doses of ^{131}I MIBG have been given to children with metastatic or recurrent tumour which had not responded to conventional chemotherapy. There is an objective response rate of about 35%³¹. The radioactive therapy has also been added early to the treatment protocol in children with advanced or inoperable neuroblastoma to reduce the tumour volume, allowing adequate surgical excision and avoiding drug toxicity and the early occurrence of drug resistance. Using this approach 70% had complete or at least >95% resection and the toxicity was less than that using the MIBG after conventional chemotherapy³².

The physical half-life of ^{131}I is 8 days and 66% of its energy is emitted as γ rays. The γ rays do not contribute significantly to the therapeutic efficacy and cause non-specific irradiation of the bone marrow and of the physical environment around the patient requiring the patient to be isolated in shielded rooms for several days. Only one third of the energy is emitted as β rays which give the main therapeutic effect and these only travel within 1 mm of the emission source. The pure β emitter ^{90}Y is more energetic than ^{131}I and is being evaluated.

The ^{123}I isotope was used in our studies and because of its short half-life, it has to be synthesized on the day of usage. The superior counting statistic of the 159 KeV photon of ^{123}I -MIBG over the 364 KeV photon of ^{131}I -MIBG means that there is a gain in diagnostic sensitivity by using the ^{123}I isotope and there is a lessening of the radiation hazard. The issue is discussed in Chapter 13.

Genes

Genetic details are outlined in Chapter 16. Mutations in different chromosomes are involved in the various familial syndromes and have also been found occasionally in sporadic cases.

The rearrangement during transfection (RET) proto-oncogene encodes a receptor tyrosine kinase function essential for the development of the kidney and intestinal nervous system.

Germline mutations affecting one of five cysteines (Cys609,611,618,620 in exon 10 and 634 in exon 11) located in the juxtamembrane domain of the RET receptor are responsible for the vast majority of MEN 2A and familial medullary carcinoma of the thyroid. These mutations result in the replacement of the cysteine by another amino acid. Mutations of RET also cause Hirschsprungs disease, a congenital aganglionosis of the hind gut. Mutations 630 and 634 activate RET more strongly than mutations at 609, 618 or 620³³. High RET transforming activity has also been reported for the aspartic acid to tyrosine (D631Y) mutation in sporadic pheochromocytomas. Further mutation analysis suggested that cysteine 630 or 634 could be involved in the disulphide-linked dimerisation induced by the D631Y mutation³⁴. Mutations in the gene at codon 618, 620 and 634 were ruled out in one recent patient in this thesis series with sporadic disease.

A single RET mutation, resulting in the substitution M918T in exon 16 has been identified in 94% of cases of MEN 2b which consists of medullary carcinoma of the thyroid, pheochromocytoma and developmental abnormalities. This mutation lies

within the substrate recognition packet of the tyrosine kinase catalytic core³⁵. Other mutations in MEN 2b have been described for example A883F³⁶

A study of the MEN 2A mutation Cys634Arg and the MEN 2B mutation Met918Thr in two RET isoforms of 1114 and 1072 amino acids showed that each RET isoform activated by MEN 2B or the MEN 2A mutation was transforming in fibroblasts and induced neuronal differentiation of pheochromocytoma PC cells in culture. Among the different RET MEN 2 mutants, the long RET isoform activated by the MEN 2B mutation stimulated the most prominent neurite outgrowth in the PC 12 cells. Morphological changes in PC12 cells caused by constitutively activated RET oncoproteins involved the engagement of a RAS-dependent pathway. This suggests that the biological properties of RET-MEN 2B mutants depend on the interplay between the RET isoforms and the nature of the activating MEN 2 mutation³⁷

In a study of 12 sporadic pheochromocytomas and 5 extra-adrenal paragangliomas, significant deletions were found in chromosomes 1p34-36 and 3p25 in sporadic pheochromocytomas but not in extra-adrenal paragangliomas³⁸. The authors' conclusion was that multiple genetic factors may be involved in pheochromocytoma tumorigenesis and that the mechanisms may differ between extra-adrenal and adrenal tumours. Further evidence in support of these conclusions comes from a Japanese study of p53 gene mutations in 33 pheochromocytomas using polymerase chain reaction-single strand conformation polymorphism. There was a relatively high incidence of p53 gene mutations or intronic sequence alteration in multiple and malignant pheochromocytomas but not in solitary cases³⁹.

In Von Hippel Lindau, about 40% of such families who also have pheochromocytoma have mutations in codon 238 in the VHL gene on chromosome 3p25.3⁴⁰. This specific investigation was not done. The incidence of pheochromocytoma in Von Hippel Lindau disease families varies from zero to 92%. Three cases, (nearly 10%) in this series were in two separate VHL families.

The index patient had bilateral pheochromocytomas first presenting at the age of 21 years and the second at two years later. She also had a right eye enucleation for Coat's Disease (a benign condition). There was extensive retinal detachment and a fleshy mass protruded from the right optic disc. She had three sons and one daughter. The son had had bilateral pheochromocytoma and there was a three year interval between presentations. This boy and two brothers have missense mutation 683 C > T, but have normal urine catecholamines and brain scans in 1999 when they were 10 and 13 years old.

The index patient in the second family had a posterior fossa haemangioblastoma removed in 1991 when aged 20 years. In 1995, she had a bilateral adrenalectomy for pheochromocytoma. Her mother died from an abdominal tumour which was not defined and her aunt, uncles and grandmother also had renal or adrenal tumours.

Another three (circa 10%) were in families with neurofibromatosis.

Details are - one 36 year old man had nine siblings. His father had a pancreatic pseudocyst, a brother had acantholytic ameloblastoma cyst of the mandible, a sister had a dermatofibroma removed from the leg, another brother had myxoid

neurofibroma of the leg, another sister had a cerebellar astrocytoma. Three had no problem.

A 30 year old had a new mutation neurofibromatosis and presented with abdominal pain, headache, coldness, facial flushing, palpitations and mild dyspnoea during an attack. He has systolic hypertension during an episode. The third patient's mother had neurofibromatosis. He has six sisters and one brother of which four sisters and the brother have neurofibromatosis.

Ganglioneuroblastoma

This is a malignant tumour composed of immature neuroblasts and mature ganglion cells in varying proportions, usually presenting in children. It is capable of producing catecholamines but symptoms from catecholamine production are rare⁴¹. Hypertension and profuse sweating have however been reported⁴². Composite pheochromocytoma and ganglioneuroblastoma has been described in a patient with MEN 2A⁴³.

Increased quantities of dopa, dopamine, noradrenaline and their metabolites may be found in the urine⁴⁴. An adrenaline-secreting ganglioneuroblastoma with elevated urinary HMMA but normal metanephrine excretion has been described⁴⁵.

References

1. Ross EJ, Griffith DNW. The clinical presentation of pheochromocytoma. *Q J Med* 1989;71:485-96.
2. deGraeff J, Horak BJV. The incidence of pheochromocytoma in the Netherlands. *Acta Med Scand* 1964;176:583-93.
3. Stenstrom G, Svarsudd K. Pheochromocytoma in Sweden 1958-81. *Acta Med Scand* 1986;suppl 220:225-32.
4. Tormey WP, Fitzgerald RJ. Lack of uniformity in the clinical approach to the interpretation of urinary catecholamines and their metabolites. *Ir J Med Sci* 1995;164:146-50.
5. Graham PE, Smythe GA, Edwards GA, Lazarus L. Laboratory diagnosis of pheochromocytoma: which analytes should we measure. *Ann Clin Biochem* 1993;30:129-34.
6. O'Neill G, Tormey WP, Farrell MA, FitzGerald RJ, Finucane J. Pheochromocytoma and lethal lactic acidemia. *Ir Med J* 1993;86:221.
7. Goldstein RE, O'Neill JA Jr, Holcomb GW 3rd, Morgan WM 3rd, Neblett WW 3rd, Oates JA, Brown N, Nadeau J, Smith B, Page DL, Abumrad NN, Scott HW Jr. Clinical experience over 48 years with pheochromocytoma. *Ann Surg* 1999;299:755-64.

8. Lumachi F, Polistina F, Favia G, D'Amico DF. Extraadrenal and multiple pheochromocytomas. Are there really any differences in pathophysiology and outcome? *J Exp Clin Cancer Res* 1998;17:303-5.
9. Orchard T, Grant CS, van Heerden JA, Weaver A. Pheochromocytoma-continuing evolution of surgical therapy. *Surgery* 1993;114:1153-8.
10. Grobmyer SR, Guillem JG, O'Riordain DS, Woodruff JM, Shriver C, Brennan MF. Colonic manifestations of multiple endocrine neoplasia type 2B:report of four cases. *Dis Colon Rectum* 1999;42:1216-9.
11. Zimlichman R, Levinson PD, Kelly G, Stull R, Keiser HR, Goldstein DS. Derivation of urinary dopamine from plasma dopamine. *Clin Sci* 1988;75:515-20.
12. Marini M, Fathi M, Vallotton M. Dosage des metanephrines seriques pour le diagnostic du pheochromocytome. *Ann Endocrinol (Paris)* 1993;54:337-42.
13. Roberts NB, Dutton J, McClelland P, Bone JM. Urinary catecholamine excretion in relation to renal function. *Ann Clin Biochem* 1999;36:587-91.
14. Smythe GA, Edwards G, Graham P, Lazarus L. Biochemical diagnosis of pheochromocytoma by simultaneous measurement of urinary excretion of epinephrine and norepinephrine. *Clin Chem* 1992;38:486-92.
15. Graham PE, Smythe GA, Edwards GA, Lazarus L. Laboratory diagnosis of phaeochromocytoma:which analytes should we measure. *Ann Clin Biochem* 1993;30:129-34.
16. Mann DL, Kent RL, Parsons B, Cooper G IV. Adrenergic effects on the biology of the adult mamalian cardiocyte. *Circulation* 19192;85:790-804.
17. Gatzoulis KA, Tolis G, Theopistou A, Gialafos JH, Toutouzas PK. Cardiomyopathy due to a pheochromocytoma. A reversible entity. *Acta Cardiol* 1998;53:227-9.
18. Mihai R, Wong NA, Luckett M, Sheffield E, Farndon JR. No correlation between phaeochromocytoma catecholamine secretion and granule ultrastructure. *Br J Surg* 1998;85:1681-5.
19. Xiao XR, Ye LY, Shi LX, Cheng GF, Li YT, Zhou BM. Diagnosis and treatment of adrenal tumours: a review of 35 years' experience. *Br J Urol* 1998;82:199-205.
20. Crout J, Sjoerdsma A. Turnover and metabolism of catecholamines in patients with pheochromocytoma. *J Clin Invest* 1964;43:94-102.
21. Lenders JWM, Keiser HR, Goldstein DS, Willemsen JJ, Friberg P, Jacobs M-C, Kloppenborg PWC, Thien T, Eisenhofer G. Plasma metanephrines in the diagnosis of pheochromocytoma. *Ann Intern Med* 1995;123:101-9.

22. Miyazawa K, Kigoshi T, Nakano S, Kobayashi Y, Suzuki K, Tsugawa R, Uchida K. Hypertension due to coexisting pheochromocytoma and aldosterone-producing adrenal cortical adenoma. *Am J Nephrol* 1998;18:547-50.
23. Hiner LB, Gruskin AB, Baluarte HJ, Cote ML, Sapire DW, Levitsky D. Plasma rennin activity and intrarenal blood flow distribution in a child with a pheochromocytoma. *J Pediatr* 1976;89:950-2.
24. de Mendonca WC, Espat PA. Pheochromocytoma associated with fibromuscular dysplasia. *Am J Clin Pathol* 1981;75:749-54.
25. Castaigne V, Afriat R, Cambouris-Perrine S, Radu S, Desdout J, Freund M. Association pheochromocytome et grossesse. A propos de 2 cas et revue de la litterature. [Pheochromocytoma associated with pregnancy. Report of 2 cases and review of the literature.] *J Gynecol Obstet Biol Reprod (Paris)* 1998;27:622-4. (in French)
26. Chatal J-F, Hoefnagel CA. Radionuclide therapy. *Lancet* 1999;354:931-5.
27. Nguyen HH, Proye CA, Carnaille B, Combenale F, Pattou FN, Huglo D. Tumour size: the only predictive factor for ¹³¹I MIBG uptake in phaeochromocytoma and paraganglioma. *Aust NZ J Surg* 1999;69:350-3.
28. Shulkin BL, Thompson NW, Shapiro B, Francis IR, Sisson JC. Pheochromocytomas: imaging with 2-(fluoro-18) fluoro-2-deoxy-D-glucose PET. *Radiology* 1999;212:35-41.
29. Sisson JC, Shapiro B, Shulkin BL, Urba S, Zempel S, Spaulding S. Treatment of malignant pheochromocytomas with ¹³¹-I metaiodobenzylguanidine and chemotherapy. *Am J Clin Oncol* 1999;22:364-70.
30. Letizia C, De Toma G, Massa R, Corsi A, Caliumi C, Subioli S, D'Erasma E. False-positive diagnosis of adrenal pheochromocytoma on iodine-123-MIBG scan. *J Endocrinol Invest* 1998;21:779-83.
31. Troncone L, Galli G. Proceedings of the international workshop on the role of [¹³¹I] metaiodobenzylguanidine in the treatment of neural crest tumours. *J Nucl Biol Med* 1991;35:177-362.
32. De Kraker J, Hoefnagel CA, Caron H, Valdes Olmos RA, Zsiros J, Heij HA, Voute PA. First line targeted radiotherapy, a new concept in the treatment of advanced stage neuroblastoma. *Eur J Cancer* 1995;31A:600-2.
33. Chappuis-Flament S, Pasini A, De Vita G, Segouffin-Carriou C, Fusco A, Attie T, Lenoir GM, Santoro M, Billaud M. Dual effect on the RET receptor of MEN 2 mutations affecting specific extracytoplasmic cysteines. *Oncogene* 1998;17:2851-61.

34. Asai N, Iwashita T, Murakami H, Takanari H, Ohmori K, Ichihara M, Takahashi M. Mechanism of RET activation by a mutation at aspartic acid 631 identified in sporadic pheochromocytoma. *Biochem Biophys res Commun* 1999;255:587-90.
35. Mulligan LM, Kwok JBJ, Healey CS et al. Germ-line mutations of the RET Proto-oncogene in multiple endocrine neoplasia. *Nature* 1993;363:458-60.
36. Smith DP, Houghton C, Ponder BA. Germline mutation of RET codon 883 in two cases of de novo MEN 2B. *Oncogene* 1997;15:1213-7.
37. Rossel M, Pasini A, Chappuis S, Geneste O, Fournier L, Schuffenecker I, Takahashi M, van Grunsven LA, Urdiales JL, Rudkin BB, Lenoir GM, Billaud M. Distinct biological properties of two RET isoforms activated by MEN 2A and MEN 2B mutations. *Oncogene* 1997;14:265-75.
38. Vargas MP, Zhuang Z, Wang C, Vortmeyer A, Linehan WM, Merino MJ. Loss of heterozygosity on the short arm of chromosomes 1 and 3 in sporadic pheochromocytomas and extra-adrenal paraganglioma. *Hum Pathol* 1997;28:411-5.
39. Yoshimoto T, Naruse M, Zeng Z, Nishikawa T, Kasajima T, Toma H, Yamamori S, Matsumoto H, Tanabe A, Naruse K, Demura H. *J Endocrinol* 1998;159:247-55.
40. Chen F, Kishida T, Yao M, Hustad T, Glavac D, Dean M, Gnarr JR, Orcutt ML, Duh FM, Glenn G. Germline mutations in the von Hippel-Lindau disease tumor suppressor gene: correlations with phenotype *Hum Mutat* 1995;5:66-75.
41. Voorhess ML. The catecholamines in tumour and urine from patients with neuroblastoma, ganglioneuroblastoma and pheochromocytoma. *J Pediatr Surg* 1968;3:147-8.
42. Rosenstein BJ, Engelman K. Diarrhea in a child with a catecholamine-secreting ganglioneuroma. Case report and review of the literature. *J Pediatr* 1963;63:217-66.
43. Matias-Guiu X, Garrastazu MT. Composite phaeochromocytoma-ganglioneuroblastoma in a patient with multiple endocrine neoplasia type 11A. *Histopathology* 1998;32:281-2.
44. Manger WM, Gifford RW. Neuroblastoma, ganglioneuroblastoma and ganglioneuroma, Chapter 5, Differential diagnosis in Clinical and experimental pheochromocytoma, 2nd ed, Blackwell Scientific, Cambridge Mass, USA 1996, 176-9.
45. Roberts NB, Dutton J, White MC, Winstanley J, Sells RA. An adrenaline-secreting ganglioneuroblastoma with elevated urinary HMMA but normal metanephrine excretion. *Ann Clin Biochem* 1992;29:678-80.

Diagnostic sensitivities and specificities of individual catecholamines and combinations which includes HMMA in the diagnosis of phaeochromocytoma.

The results have been published in the Irish Journal of Medical Science 1995;164:146-150. The detailed data were not listed. These data relate to the years 1989 to 1994 inclusive.

For noradrenaline, there were 27 true positives, and 7 false negatives giving a sensitivity of 79.4%. There were 938 true negatives and 39 false positives resulting in a specificity of 96%.

For adrenaline, there were 17 true positives and 16 false negatives giving a sensitivity of 51.5%. There were 912 true negatives and 65 false positives resulting in a specificity of 93.3%.

For dopamine, there were 4 true positives and 29 false negatives giving a sensitivity of 12%. There were 938 true negatives and 39 false positives resulting in a specificity of 96%.

For all three catecholamines together, there were 28 true positives and 6 false negatives giving a sensitivity of 82%. There were 864 true negatives and 113 false positives resulting in a specificity of 88.4%.

For HMMA alone, there were 21 true positives and 14 false negatives, giving a sensitivity of 60%. There were 2684 true negatives and 116 false positives resulting in a specificity of 95.8%.

For a combination of all three catecholamines and HMMA together, there were 28 true positives and 6 false negatives, giving a sensitivity of 82%. There were 2609 true negatives and 191 false positives giving a specificity of 93.2%.

Predictive values

The predictive value of a positive urinary noradrenaline, indicating the percentage of patients with a positive test who have the disease was 40.9% calculated by

$$\text{true positives} / \text{true positives plus false positives} \times 100.$$

The predictive value of a positive urinary adrenaline was 20.7%, urinary dopamine 9.3% and HMMA 15.3%.

The predictive value of a negative test which is the percentage of patients with negative tests who are not diseased is the

$$\text{true negatives} / \text{true negatives plus the false negatives} \times 100.$$

The predictive value of a negative urinary noradrenaline in the diagnosis of phaeochromocytoma was 99.25%; for urinary adrenaline, it was 98.27%; for dopamine it was 97% and for HMMA it was 99.48%.

Efficiency

This is the percentage of patients correctly classified as diseased or non-diseased and

$$= \frac{\text{true positives} + \text{true negatives}}{\text{true positives} + \text{false positives} + \text{false negatives} + \text{true negatives}} \times 100$$

For urinary noradrenaline, this is 95.45%; for urinary adrenaline it is 92%; for urinary dopamine it is 93.26% and for HMMA, it is 95.41%.

For the urinary adrenaline, noradrenaline and dopamine together, it is 88.23% and for all three catecholamines together plus HMMA, it is 93%.

Published performances of biochemical indices in phaeochromocytoma diagnosis

Bravo et al 1979 Cleveland Clinic, USA¹.

Plasma catecholamines	-	Sensitivity 95.6%
Urinary HMMA	-	Sensitivity 50%
Metanephrines	-	Sensitivity 77.3%
HMMA + Metanephrines	-	Sensitivity 86.4%

Reference values total plasma catecholamines 260 – 620 ng/L (1.54 – 3.66 nmol/L) in controls and 550 – 1405 ng/L (3.25 – 8.30 nmol/L) in 195 hypertensives expressed as +/- 3SD of the mean.

Methods: Plasma catecholamines measured by radioenzymatic assay; urinary parameters by the Pisano colorimetric methods.

Reference values for HMMA 0.7 – 6.8 mg/ 24 hr (3.53 – 34.31 µmol) and for metanephrine 0.3 –0.9 mg (1.58 – 4.74 µmol/24 hr). Derivation not stated.

Data from 24 patients with phaeochromocytoma and 40 patients suspected of the disease.

Moyer et al 1979 Mayo Clinic, Rochester New York².

Urine noradrenaline,adrenaline and dopamine together sensitivity 97% specificity 100%		
Urine metanephrines	-	sensitivity 95%
noradrenaline	-	sensitivity 91%
adrenaline	-	sensitivity 45.5%
dopamine	-	sensitivity 45.5%

(Reference values – Urine metanephrines <1.3 mg/24 hr (<6.63 µmol/24 hr); noradrenaline <80 µg/24 hr, adrenaline <20 µg/24 hr, dopamine <400 µg/24 hr)

Data from 22 patients with phaeochromocytoma and 85 essential hypertensives.

Method: HPLC with amperometric electrochemical detection.

Plouin et al 1981 Paris, France³

Urinary HMMA	
metanephrines	
noradrenaline and adrenaline	Sensitivity 100%
Plasma adrenaline and noradrenaline	
Sensitivity 80%	

(Reference values HMMA 7.56 – 32.3 mmol/24 hr; metanephrines 0.84 – 3.24 mmol/24 hr; adrenaline + noradrenaline 0.30 – 0.64 mmol/24 hr. Plasma adrenaline 0.10 – 2.03 nmol/L, plasma noradrenaline 0.24 – 3.35 nmol/L.
15 patients with phaeochromocytoma and 35 hypertensive controls

Method- Plasma levels measured by radioenzymatic assay. Method for urine parameters not stated.

Bravo and Gifford – Cleveland Clinic, 1984⁴.

Plasma noradrenaline + adrenaline	sensitivity 94%	specificity 97%
(ref <950 pg/ml)		
Urinary metanephrines	sensitivity 79%	specificity 93%
(ref <1.8 mg/24 hr, 9.7 µmol/day)		
HMMA	sensitivity 42%	specificity 100%
(ref <11 mg/24Hr, 55 µmol/day)		

Reference values are the 95% upper confidence limits in 30 essential hypertensives.
Results calculated from 43 patients with phaeochromocytoma.

Manu and Runge 1984, USA⁵

These authors exhaustively searched the literature of the time and found nine papers in English where data was reported from at least 15 patients with phaeochromocytomas and details of control groups. For each test the sensitivity-specificities were “transformed to express the mathematical indicator of its received operating characteristic curve (ROC) ie discrimination index. The discrimination index values allow ranking of the tests from best (greatest discrimination index) to worst according to the difference in their total distributions.” Different sensitivities and specificities can then be related.

The urinary metanephrines appeared to be the best test.

	Number of patients	specificity	sensitivity	di
Urinary HMMA	294	0.99	0.84	3.32
Urinary Metanephrines	282	0.99	0.96	4.07
Urinary Catecholamines	179	0.99	0.85	3.36

di = discrimination index

Data from references 1, 3 shown here formed part of the study. An earlier report of the Bravo and Gifford data was also included. It is unclear that all the data used was independent because three papers had overlapping authorship (refs 8,9,10 in Manu and Runge)

Januszewicz and Wocial, 1985 Warsaw, Poland⁶.

Urinary noradrenaline	sensitivity 30%
Urinary noradrenaline plus adrenaline	sensitivity 44%
But there is ambiguity and contradiction between the illustration in the paper and the text with the text giving a sensitivity of 91% for the combination of urinary noradrenaline and adrenaline.	
Urinary dopamine	sensitivity 44.4%
Urinary HMMA	sensitivity 8% again this figure is ambiguous and contradictory as there are only 30% normal values shown for HMMA.
Urinary normetanephrine and metanephrine	sensitivity 30% but again only about 4% are shown as normal in the same table.
Urinary normetanephrine	sensitivity 27%

Data from 110 patients with phaeochromocytoma from 1965 to 1984.

Reference values are not stated.

Methods: early methods measured free urinary catecholamines by fluorimetry. Later HMMA and total metanephrines measured by fluorimetry and colorimetry. Later fluorimetric methods were introduced for plasma noradrenaline, adrenaline, urinary dopamine, DOPA and 3-methoxy-4-hydroxyphenylglycol (MHPG). Then radioenzymatic methods for plasma catecholamines and urinary conjugated catecholamine.

Duncan et al 1988 Australia⁷

Urine noradrenaline	sensitivity 100%	specificity 100%
Noradrenaline/ Dihydroxyphenylglycol ratio	sensitivity 100%	specificity 99%
Plasma noradrenaline	sensitivity 82%	specificity 95%

Reference – Both plasma and urinary noradrenaline values were skewed and were normalized by log transformation. The upper limit was taken as the 95% confidence limit after log transformation. Urine noradrenaline 95% confidence interval from 66 to 924 nmol/24 hr, from 1010 hypertensive patients without tumour. Plasma free noradrenaline

0.83 to 10.0 nmol/L in 263 samples from the same population. Data was calculated from 25 phaeochromocytoma patients from 1086 hypertensive patients.

Methods were combined gas chromatography and mass spectr ometry.

Oishi et al, Kumamoto, Japan. 1988⁸.

Using 24 hour urinary collections in glass bottles containing 20 ml 6mol/L HCL

	sensitivity
Urinary normetanephrine	100%
Urinary metanephrene	87.1%
Urinary total metanephtrines	100%
Urinary noradrenaline	90.3%
Urinary adrenaline	80.6%
Urinary adrenaline plus noradrenaline	93.5%

Data from 31 patients with phaeochromocytoma in 24 hour urine collections

Randomly voided 1 hour urine specimens were taken in the morning following bed rest in 24 patients with phaeochromocytoma, 31 with essential hypertension and 16 normal subjects. Subjects were asked to void before bed rest and that sample was discarded. Then the next sample was taken into a glass bottle containing 3 ml 2 mol/L HCL.

1-hour values resulted in

	sensitivities
Urine normetanephrine	100%
Urine metanephrene	75%
Urine total metanephtrines	100%
Urine noradrenaline	87%
Urine adrenaline	71%
Urine noradrenaline + adrenaline	87%

Data was calculated from 40 patients with essential hypertension and 30 patients who had phaeochromocytoma excluded. 3 SD above the mean for normal subjects was taken as the upper reference limit. The authors seem to have treated the data as being normally distributed. Normals noradrenaline <343, adrenaline <51, noradrenaline plus adrenaline <376, normetanephrine <1830, metanephrene <992, total metanephtrines < 2626, all units in nmol/day.

Method: urinary metanephrene and normetanephrene by radioimmunoassay and urine catecholamines by fluorimetric measurement..

Mornex et al, Hopital Eduard Herriot, Lyon, France 1991⁹.

Plasma normetanephrine	sensitivity	96%
Plasma metanephrine	sensitivity	69%
Plasma normetanephrine and metanephrine	sensitivity	98%

Values were very high in four patients with renal failure.

Values for urinary metanephrines were shown in 16 patients with phaeochromocytoma.

Urinary total metanephrines sensitivity 100% using either hypertensive patients or normal subjects as the reference.

Reference range from 26 healthy subjects, 33 patients with essential hypertension and 14 with miscellaneous diseases.

Reference values were 0.40+/- 0.10 ng/ml for plasma metanephrine and 0.85 +/- 0.25 ng/ml for plasma normetanephrine. The sum of plasma normetanephrine and metanephrine was 1.25 +/-0.28 and the range was 0.9 to 1.9 ng/ml. Values of the total plasma metanephrines in the essential hypertensives were 1.2 to 6.0 ng/ml. Values of the total plasma metanephrines in phaeochromocytoma patients ranges from 6.2 to 436 ng/ml. Urinary metanephrine plus normetanephrine in healthy subjects was 0.2 – 0.6 mg/day. In essential hypertensives, urinary metanephrine plus normetanephrine was 0.2 – 0.9 mg/day. In the phaeochromocytomas, the values were 1.3 – 32 mg/day.

Data taken from 49 samples from 22 patients with phaeochromocytoma.

Method Plasma analytes were measured by HPLC with electrochemical detection preceded by a hydrolysis by heating and purification by Dowex 50W x 2, column. Urinary metanephrines were assayed by specific fluorimetry after purification through a double-step ion-exchange procedure.

Ross et al St Bartholomew's Hospital, London 1993¹⁰

Urinary noradrenaline	sensitivity 100%	specificity 99.5%
Plasma catecholamine	sensitivity 85%	specificity 97.5%
noradrenaline	sensitivity 84.6%	
adrenaline	sensitivity 15.4%	

Reference ranges: Urine noradrenaline 50 – 571 nmol/24 hr; adrenaline 8 – 149 nmol/24 hr; dopamine 0 –3237 nmol/24 hr. Plasma noradrenaline 0.47 – 4.12 nmol/L; adrenaline 0.03 – 1.31 nmol/L (mean + 2 sd). Plasma noradrenaline and adrenaline were skewed and approximated to a normal distribution on logarithmic transformation.

Data calculated on plasma catecholamines from 545 patients and urine catecholamines on 106 patients. There were 13 phaeochromocytomas in the plasma group and 11 had urinary investigations.

Methods: All used HPLC with electrochemical detection.

Graham et al Australia 1993¹¹

Urine noradrenaline	Sensitivity 70%	Specificity 98%
Adrenaline	50%	98%
Noradrenaline and adrenaline	100%	97%
Normetadrenaline	90%	98%
Metadrenaline	70%	99%
Normetadrenaline and metadrenaline	100%	98%
HMMA	70%	98%

Reference values urine (all per 24 hr) noradrenaline 900 nmol, adrenaline 190 nmol, dopamine 5200 nmol, normetadrenaline 5.1 µmol, metadrenaline 2.0 µmol, HMMA <48 µmol (upper 95th percentile). Values derived from 20 patients with phaeochromocytoma and from a non-tumour hypertensive population varying from 2476 collections for noradrenaline to 300 for HMMA.

Method: gas chromatography-mass spectrometry for noradrenaline, adrenaline, dopamine and 3,4-dihydroxyphenylglycol. HPLC and electrochemical detection was used for HMMA, normetadrenaline and metadrenaline.

Lenders et al 1993, NIH Maryland USA¹²

Plasma normetadrenaline	Sensitivity 100%
Plasma metadrenaline	Sensitivity 43%

Reference values, plasma normetanephine 0.12 – 0.73 (0.34 +/- 0.16) nmol/L. Plasma metanephine 0.06 – 0.20 (0.13 +/- 0.04) nmol/L from 14 normal subjects and 18 in-patients.

Data from 23 patients with phaeochromocytoma

Method: HPLC with electrochemical detection.

Neumann et al 1993, Freiburg, Germany¹³.

Data from screening 79 subjects at risk for phaeochromocytoma who were members of families with MEN-2 or Von Hippel Lindau disease and their first degree relatives, patients with retinal angiomas as their only symptom and first degree relatives of patients with multifocal phaeochromocytomas.

Urinary noradrenaline	Sensitivity 86%	Specificity 95%
Urinary adrenaline	Sensitivity 53%	Specificity 98%
Urinary HMMA	Sensitivity 64%	Specificity 91%
Plasma noradrenaline	Sensitivity 58%	Specificity 97%
Plasma adrenaline	Sensitivity 33%	Specificity 94%
Plasma chromogranin A	Sensitivity 52%	Specificity 95%

Reference values- The upper reference limits were urinary noradrenaline 81 ug/day (479 nmol/day), adrenaline 14 ug/day (76.4 nmol/L), HMMA 7 mg/day (35.3 µmol/day). Plasma limits were noradrenaline 0.73 ng/ml (4.31 nmol/L) , adrenaline 0.13 ng/ml (0.71 nmol/L), chromogranin A 55 ng/ml.

Methods: Plasma noradrenaline and adrenaline were measured radioenzymatically, Urinary noradrenaline, adrenaline and HMMA by spectrofluorometry., plasma chromogranin A by radioimmunoassay.

Marini et al, Geneva, Switzerland. 1993¹⁴.

Plasma metanephrines Sensitivity 100%* Specificity 94%*

The above performances relate to values in phaeochromocytoma above a threshold of 50 nmol/L and only when renal function is normal.

The range of total metanephrine values found with phaeochromocytoma was 76 to 718 nmol/L. The 18 healthy controls had values from 0.5 to 10.7 nmol/L. Values in 33 essential hypertensives ranged from 7 to 138 nmol/L and values in 11 patients undergoing haemodialysis twice or three times weekly for chronic renal disease were 188 to 920 nmol/L. The mean values of the latter was 471 nmol/ l while the man for the patients with phaeochromocytoma was 326 nmol/L. Gaussian statistics were inappropriately used as the data was clearly skewed. However, it is clear that there is an overlap between the patients with essential hypertension and those with phaeochromocytoma or with renal failure.

Urinary metanephrines were determined in 8 of the 10 patients with phaeochromocytoma in 24 hour urines containing 15 ml HCL 6 M. . The values ranged from 9 to 268 µmol/day. All values were elevated. Post-operative urinary metanephrine values ranged from 1.7 to 4.0 µmol/day.

Urinary metanephrines sensitivity 100%

Reference values in urine are not stated, however a value <4.1 µmol/day would separate all the pre-operative tumour values from the post-operative values.

Data calculated from 10 patients with phaeochromocytomas.

Method: HPLC with amperometric electrochemical detection.

Gerlo et al Belgium 1994¹⁵.

Reference values for urinary catecholamines corresponded to the 97.5 percentiles of a population which included hypertensives. The reference values for urinary metanephrines were determined from a similar population of 60 men and 105 women. The reference values for plasma catecholamines were taken from a paper by Hamberger et al 1981¹⁶.

	Sensitivity	Upper reference limit
Urine noradrenaline	73.4%	males 0.63, female 0.47 µmol/day
Urine adrenaline	78.9%	males 0.099, female 0.074 µmol/day
Urine normetanephrine	83.3%	males 4.4, female 3.3 µmol/day
Urine metanephrine	100%	males 1.8, females 1.2 µmol/day
Urine HMMA	84.2%	males/females 33 µmol/day
Plasma noradrenaline	50%	males/females 5 µmol/L
Plasma adrenaline	60%	males/females 1 µmol/L

Data collected from urinary collections in 19 patients with phaeochromocytoma and plasma catecholamine levels were taken from 10 of these. False positive values were not included so specificities could not be calculated.

Methods: HPLC with electrochemical detection

Beaumont Study 1995¹⁷

Urinary Noradrenaline	Sensitivity 79%	Specificity 96%
Adrenaline	51.5%	93.3%
Dopamine	12%	96%
Noradrenaline + adrenaline + dopamine	82%	88.4%
HMMA	60%	95.8%
Catecholamines and HMMA	82%	93.2%

Data for these calculations follows in this chapter.

Method: HPLC with electrochemical detection.

Lenders et al National Institutes of Health Maryland, USA, Nijmegen Holland and Goteborg, Sweden 1995¹⁸

Plasma

Normetadrenaline plus metadrenaline	Sensitivity 100%	Neg predictive value 100%
	Specificity 84.8%	Pos predictive value 64.2%

Normetanephine (alone)	Sensitivity 98%
------------------------	-----------------

Total metanephines	Sensitivity 98%	Pos predictive value 63%
		Neg predictive value 99%

Plasma noradrenaline plus adrenaline	Sensitivity 84.6%	Neg predictive value 95%
	Specificity 81.6%	Pos predictive value 56%

<i>Urine</i> metanephines	Sensitivity 89%
----------------------------------	-----------------

24 hour urine collections were made in 46 of the 52 patients with phaeochromocytoma. Five of 46 patients had false negative urine metanephines but all 5 had increased plasma metanephines whereas only 3 had increased plasma catecholamines. False positive plasma catecholamines occurred in 35 (18%) of 191 patients without catecholamines. In 19 of these 35, the plasma metanephines were also false positive.

False positive tests for plasma metanephine 15%
False positive tests for plasma catecholamines 18% .

Data from 52 patients with phaeochromocytoma of which 13 had malignant tumours. Plasma values measured by HPLC but urine values by colorimetric Pisano method. Reference values from a group of 51 patients with essential hypertension and 67 healthy normotensives. A false negative result in a patient with phaeochromocytoma was defined where both normetanephine and metanephine were below their upper reference limits. Noradrenaline and adrenaline were also treated as a pair. However a false positive result for plasma metanephines or catecholamines was where any individual result was equal to or above the upper respective reference limit in a patient without phaeochromocytoma.

The upper reference limits were 0.66 nmol/L for plasma normetanephine, 0.30 nmol/L for metanephine, 3.00 nmol/l for noradrenaline and 0.54 nmol/L for adrenaline. For urinary metanephines, the upper reference limit of 6.8 µmol/24 hour derived from normotensive patients was used.

Note on reference values:- Normal distributions of plasma catecholamines and metanephines were obtained after logarithmic transformation of the data from 67 normotensive and 51 hypertensives. The upper reference limit was defined as the 97.5th

percentile. This was calculated from the anti-logarithm of the mean plus 2 standard deviations of the transformed data.

Heron E et al – Paris, France 1996¹⁹.

Urinary metanephrine	Sensitivity 95%	Specificity 98%
	Positive predictive value 46%	
	Negative predictive value 100%	
Urinary metanephrine to creatinine ratio		
	Sensitivity 100%	Specificity 98%
	Positive predictive value 47%	
	Negative predictive value 100%	

Data from 20 patients with phaeochromocytoma and from a total of 1013 patients which included data from 16 patients considered disease-free following phaeochromocytoma resection. Method by HPLC with electrochemical detection.

Reference values: Urinary metanephrine 3.70 µmol/24 hours corresponding to the mean + 2.23 SDs of the values in the study sample. The mean + 2.23 SDs of the metanephrine-to-creatinine ratio 0.354 µmol/mmol creatinine was used as the upper reference limit for that variable.

The values found from the 993 patients without phaeochromocytoma show a metanephrine level 1.95 +/- 0.92 umol/day in men and 1.55 +/- 0.80 umol/day in women – significantly different (p<0.001). Creatinine levels were 13.4 +/- 4.1 mmol/day in men and 9.0 +/- 2.9 in women (p<0.001). Metanephrine-to-creatinine ratios in men were 0.152 +/- 0.074 compared to 0.181 +/- 0.090 in women (p<0.001).

Peaston et al, Newcastle upon Tyne, England. 1996²⁰

24 hour urinary noradrenaline –	sensitivity 88%,	specificity 82%.
Urinary adrenaline -	78%	89%
Urinary dopamine -	28%	70%
Urinary normetanephrine	93%	67%
Urinary metanephrine	78%	96%
Urinary HMMA	82%	90%

Reference values Noradrenaline <610 , adrenaline <190 nmol/day: normetanephrine <2.8, metanephrine <2.2 µmol/day. Dopamine and HMMA not stated.

Values in the following study are related to creatinine in morning specimens

Overnight urinary noradrenalines were 86 – 1552 nmol/mmol creatinine (normal 14 –63)

Overnight urine	sensitivity	specificity
Noradrenaline	100%	98%
Adrenaline	73%	98%
Dopamine	25%	97%
Normetanephrine	100% (98.7%)	99%
Metanephrine	73% (37.5%)	98%
HMMA	78%	100%

Plasma values were obtained in 10 patients with phaeochromocytoma.

Plasma noradrenaline sensitivity 80%

Plasma adrenaline sensitivity 20%

Reference values plasma noradrenaline <5.0, adrenaline <1.8 nmol/L

Details on false positive (FP) values are scanty. There were 4 FPs for overnight noradrenaline among the 166 hypertensives and 2 FPs for adrenaline. There was one false negative normetanephrine/creatinine value in 76 urine overnight values from the 16 patients with phaeochromocytoma, making a sensitivity of 98.7% not the 100% stated in the published table and shown above. It is also stated in the published text that “in 10 of the 16 patients with pheochromocytoma overnight values were not significantly different from the results of the control group”. This means that there were 10 false negatives and 6 true positives ie a sensitivity of 37.5%. However the published table shows a sensitivity of 73%. HMMA levels were highly variable in 10 patients with phaeochromocytoma. There were no false positives HMMA values. There were a total of 90 samples listed for the overnight collections among the 16 phaeochromocytoma patients. But it is stated that only the initial sample was used for calculation of the sensitivities and specificities ie the bulk of the data was excluded. The Beaumont data (thesis chapters 6 and 7) does not exclude any result and are more valid.

Reference range from 166 patients with hypertension and 24 healthy normals.

The distribution of data from each group was markedly skewed and all data was logarithmically transformed to normality before statistical analysis. Then >2 SD above the mean value was the upper reference cut-off. For plasma catecholamines, the reference ranges were based on values from hypertensive patients and the upper limit was set at >2 SD from the mean.

Data from 16 patients with phaeochromocytoma

Method: Urine and plasma by HPLC with electrochemical detection with various pretreatments.

Loh K-C et al – Halifax, Nova Scotia, Canada 1997²¹.

Urinary HMMA Sensitivity 89% (in 18 patients)

Catecholamines	100% (in 14 patients)
Metanephrines	100% (in 6 patients)

Plasma noradrenaline	Sensitivity 100% (21 patients tested)
Adrenaline	46%
Dopamine	15%

(Reference ranges Urinary free catecholamines – noradrenaline <100 µg/24 hr or <591 nmol/24 hr; adrenaline <15 µg/day or <82 nmol/day; dopamine 65 – 400 µg/24 hr or 424 – 2612 nmol/24 hr); metanephrines <1.2 mg/day (<6.5 µmol/day); HMMA 2 – 7 mg/day (10 – 35 µmol/day).

Plasma noradrenaline 110 –410 pg/ml or 601 – 2239 pmol/L; adrenaline <50 pg/ml or <273 pmol/L; dopamine <87 pg/ml or <475 pmol/L.)

Data from 18 patients with phaeochromocytoma.

Methods: HMMA was measured using a modified Pisano fluorometric method for early patients and then HPLC with electrochemical detection. Urine metanephrines were measured by a fluorometric method. Urine and plasma catecholamines were measured by HPLC with electrochemical detection.

McNeil and Woollard, Auckland, New Zealand 1997²²

Urinary noradrenaline	Sensitivity 88.9%
Urinary adrenaline	Sensitivity 44.4%
Urinary dopamine	Sensitivity 50%
Urinary HMMA	Sensitivity 81.25%

Urinary noradrenaline, adrenaline and dopamine combined – specificity 98.3%

Details derived from text- false positives 41, false negatives 4, true negatives 2433.

14 of 18 (77.8%) had raised levels of either adrenaline and/or dopamine.

One case secreted adrenaline alone and one had no raised catecholamine values.

About 10% of 2490 24-hr collections from 1957 patients had a raised value in one or more catecholamines.

46 patients had values greater than twice the upper limit of normal.

Data from 18 patients with phaeochromocytoma

Methods: HPLC with electrochemical detection.

Reference limits: the values used were a multiple of twice the upper limit of normal to reduce the problem of nonspecific events.

Urine noradrenaline $\geq 1.0 \mu\text{mol/day}$ (1000 nmol/day), adrenaline $\geq 0.2 \mu\text{mol/day}$ (200 nmol/day), dopamine $\geq 6.0 \mu\text{mol/day}$ (6000 nmol/day)

Lucon et al 1997, Brazil²³.

	Sensitivities
Urinary HMMA	90%
Metanephrines	97%
Metanephrines/mg creatinine	97%
Noradrenaline	93%
Adrenaline	64%
Dopamine	66%
Serum Noradrenaline	93%
Adrenaline	67%
Dopamine	63%

Data from 50 patients with phaeochromocytoma. Specificities not published.
Reference values Urinary HMMA 2 – 12 mg/24 hr, metanephrines 0.05 – 1.2 $\mu\text{g/mg}$ creatinine, adrenaline 0.5 – 20 $\mu\text{g/day}$, noradrenaline 14 – 80 $\mu\text{g/day}$, dopamine 65 – 400 $\mu\text{g/day}$.

Plasma noradrenaline 40 – 268 pg/ml, adrenaline 0 – 75 pg/ml, dopamine 0 – 83 pg/ml.
Reference populations not stated. Laboratory methods not defined.

Eisenhofer G et al, National Cancer Institute, National Heart Lung and Blood Institute, NIH, Bethesda Maryland, USA and Nijmegen, Holland. 1999²⁴.

In the context of the detection of phaeochromocytoma in high risk patients with Von Hippel Lindau and Multiple Endocrine Neoplasia (based on proven gene mutations).

Plasma normetanephrine and metanephrine

Sensitivity 97% Specificity 96% Neg predictive value 98%
Pos predictive value 95%

Plasma Noradrenaline and adrenaline

Sensitivity 71% Specificity 86% Neg predictive value 80%
Pos predictive value 79%

Urinary Noradrenaline and adrenaline

Sensitivity 74% Specificity 96% Neg predictive value 82%
Pos predictive value 93%

Urinary metanephrines

Sensitivity 65% Specificity 95% Neg predictive value 76%
Pos predictive value 92%

Urinary HMMA

Sensitivity 47% Specificity 100% Neg predictive value 69%
Pos predictive value 100%

Methods: Plasma noradrenaline, adrenaline, normetanephrine and metanephrine and urine catecholamines were measured by HPLC with electrochemical detection. Urine metanephrines and HMMA were measured by spectrofluorometry.

Reference range; Plasma values for each analyte were measured in 125 normal subjects and 53 hypertensives. The data was logarithmally transformed to achieve a normal distribution. The upper reference limits were taken as antilog of the mean + 2 SDs of the transformed data. The upper limits for the urine values were taken from a reference laboratory.

Upper reference values used were Plasma noradrenaline 498 pg/ml (2943.2 pmol/L), plasma adrenaline 83 pg/ml (453.2 pmol/L), plasma normetanephrine 112 pg/ml (611.5 pmol/L), plasma metanephrine 61 pg/ml (421.6 pmol/L). Urinary noradrenaline 80 ug/day (472.8 nmol/day), urinary adrenaline 20 ug/day (109.2 nmol/day), urinary metanephrines 1.2 mg/day (6.3 µmol/day), urinary HMMA 7.9 mg/day (40 µmol/day).

John et al, Zurich 1999²⁵

The data from this report shows that urinary HMMA was 100% sensitive for pheochromocytoma using a reference limit of <36 µmol/day. The detail is insufficient to calculate other indices on urinary catecholamines and the analytical methods are unstated. The results are discussed in chapter 22 under 'malignancy'.

Goldstein et al Vanderbilt University, Nashville, Tennessee, USA. 1999²⁶

This is a surgical report on 108 pheochromocytomas treated from 1950 to 1998. Biochemical data is minimal.

Urinary metanephrines and HMMA sensitivity of 98%
Urinary metanephrines sensitivity 92%

Mediavilla Garcia et al, Granada, Spain. 1999²⁷.

	sensitivity
Urinary adrenaline	50%
Urinary noradrenaline	50%
Urinary metanephrines	57.9%
Urinary HMMA	50%
Urinary catecholamines and metabolites	82.35%
Plasma adrenaline	80%
Plasma noradrenaline	26.7%
Plasma catecholamines sensitivity	35.7%

Data from 20 patients with phaeochromocytoma, 9 adrenal and 11 extraadrenal. Reference values: plasma noradrenaline 135 – 300, adrenaline 20 – 60 pg/ml. Urinary noradrenaline 90 – 400 µg/day (0.54 – 2.4 µmol/day) HMMA 1 – 8.1 mg/day (5 – 40.5 µmol/day) and metanephrines 0.05 – 0.64 mg/day (0.255 – 3.26 µmol/day). Data on false positives not stated.

Methods: metanephrines by spectrophotometric assay, HMMA by enzymeimmunoassay and catecholamines by HPLC with electrochemical detection.

Mannelli at al, Italy 1999²⁸

	sensitivity
Urinary HMMA	73.7%
Urinary noradrenaline	78.6%
Urinary adrenaline	65.8%
Urinary total catecholamines	86%
Plasma noradrenaline	82.7%
Plasma adrenaline	65.1%
Plasma noradrenaline and adrenaline	88.7%

There are no data on false positive results and neither are there data on reference ranges used to determine the above sensitivities.

Data from 284 patients with phaeochromocytoma between 1978 and 1997 in 18 centres.

Tormey – Current Thesis The sensitivities for phaeochromocytoma diagnosis from 1989 to September 1999 inclusive are described in the Results and Case Details chapter 6.

The Australian group reported three times on their figures from 1988 to 1993 as their studies evolved ^{7,11,29}. Both papers by Lenders et al are from the same group ^{12,18} as were

those from the Cleveland Clinic^{1,4}. There were also two papers from the French group^{3,19}. The purpose of the second paper from France¹⁹ is to try to address the error problem of false positive and false negative results from over and under collection periods in 24 hour urine collections. Accurate calculation of the specificities was not possible in most of these reports as the numbers of false positives and true negatives were not listed. The findings in the paper from Poland are confusing because the text indicates that the diagnostic sensitivity of urinary free catecholamines was 91% whereas the illustration of analyte sensitivity shows a maximum of 43% approximately for any analyte⁶.

Excluding inconvenient data from calculations.

In calculating sensitivities and specificities in the Beaumont study¹⁷, all analytical data was included. However, careful analysis of the data in the Nova Scotia study²¹ shows that five patients had normal as well as raised values for urinary HMMA. These normal values were not included in the published sensitivity calculations. This was also a feature of urinary catecholamine results from two patients where the sensitivity is given as 100%. Recalculating from their table, making the assumption that every urine collection on each patient is listed, the sensitivity for urine catecholamines is 93% with two false negatives in 29 samples. The exclusion of data which may mislead clinically is invalid in statistical calculations. The Belgian paper from Gerlo et al found a considerable sensitivity advantage for urinary metanephrines over the catecholamines¹⁵. Data exclusion is also a problem with the data from Peaston et al²⁰.

Reference limits

Direct comparisons of these studies must take into account the differences in reference ranges which have a great bearing on the number of positive results both true and false. The stated reference values for urinary HMMA and metanephrines in the published Cleveland Clinic data of 1979 and 1984 from Bravo et al^{1,4} differ considerably. The improvement in the diagnostic sensitivity of urinary metanephrines from 77.3% to 79% is surprising considering that the upper reference limit more than doubled. Graham et al¹¹ showed the effects of lowering the reference limits in the diagnosis of pheochromocytoma. At a HMMA cut-off of <48 µmol/day, the sensitivity is 70% and the specificity 98%. When the cut-off is reduced to 35 and 30 µmol/day, the sensitivities are 100% but the specificities fall to 92% and 85% respectively. The receiver operator curves of Lenders et al take the different reference limits into account and show that plasma metanephrines are diagnostically superior to plasma catecholamines¹⁸. The data from Marini et al¹⁴ on plasma metanephrines shows a large overlap between patients pheochromocytoma and those with essential hypertension or chronic renal failure and suggests that the value of plasma metanephrines in pheochromocytoma diagnosis requires further study. The four patients with renal failure referred to by Mornex et al⁹ support the latter observation. The relatively low reference values used by Neumann et al¹³ contributed to the success of the catecholamine screen in patients at risk for unsuspected pheochromocytomas in families with Von Hippel Lindau disease or with Multiple Endocrine Neoplasia type2. Low reference values largely derived from normotensive subjects were also used by Eisenhofer et al in addressing a similar clinical

problem²⁴. Analytical performance with information on between-batch and within-batch measures of imprecision is relevant in comparing diagnostic performances. The performance of individual analytes in external quality assessment schemes is also important because many laboratories derive some of their reference values from the literature and the results of external quality assessment schemes act as 'de facto' target values for laboratories.

References

1. Bravo EL, Tarazi RC, Gifford RW, Stewart BH. Circulating and urinary catecholamines in pheochromocytoma. Diagnostic and pathophysiologic implications. *N Engl J Med* 1979;301:682-6.
2. Moyer TP, Jiang N-S, Tyce GM, Sheps SG. Analysis of urinary catecholamines by liquid chromatography with amperometric detection: methodology and clinical interpretation of results. *Clin Chem* 1979;25:256-63.
3. Plouin PF, Duclos JM, Menard J, Comoy E, Bohuon C, Alexandre JM. Biochemical tests for diagnosis of phaeochromocytoma: urinary versus plasma determinations. *Br Med J* 1981;282:853-4.
4. Bravo EL, Gifford RW. Pheochromocytoma : diagnosis , localization and management. *N Engl J Med* 1984;311:1298-1303.
5. Manu P, Runge LA. Biochemical screening for pheochromocytoma- superiority of urinary metanephrines measurements. *Am J Epidemiol* 1984;120:788-90.
6. Januszewicz W, Wocial B. Clinical and biochemical aspects of pheochromocytoma. Report of 110 cases. *Cardiology* 1985;72:suppl1:131-6.
7. Duncan MW, Compton P, Lazarus L, Smythe GA. Measurement of norepinephrine and 3,4-dihydroxyphenylglycol in urine and plasma for the diagnosis of pheochromocytoma. *N Engl J Med* 1988;319:136-42.
8. Oishi S, Sasaki M, Ohno M, Sato T. Urinary normetanephrine and metanephrine measured by radioimmunoassay for the diagnosis of pheochromocytoma: utility of 24-hour and random 1-hour urine determinations. *J Clin Endocrinol Metab* 1988;67:614-8.
9. Mornex R, Peyrin L, Pagliari R, Cottet-Emard JM. Measurement of plasma methoxyamines for the diagnosis of pheochromocytoma. *Horm Res* 1991;36:220-6.
10. Ross GA, Newbound EC, Thomas J, Bouloux PMG, Besser GM, Perrett D, Grossman A. Plasma and 24h-urinary catecholamine concentrations in normal and patient populations. *Ann Clin Biochem* 1993;30:38-44.

11. Graham PE, Smythe GA, Edwards GA, Lazarus L. Laboratory diagnosis of pheochromocytoma: which analytes should we measure. *Ann Clin Biochem* 1993;30:129-34.
12. Lenders JWM, Eisenhofer G, Armando I, Keiser HR, Goldstein DS, Kopin IJ. Determination of metanephrines in plasma by liquid chromatography with electrochemical detection. *Clin Chem* 1993;39:97-103.
13. Neumann HPH, Berger DP, Sigmund G, Blum U, Schmidt D, Parmer RJ, Volk B, Kirste G. Pheochromocytomas, multiple endocrine neoplasia type 2, and von Hippel-Lindau disease. *N Engl J Med* 1993;329:1531-8.
14. Marini M, Fathi M, Vallotton M. Determination of serum metanephrines in the diagnosis of pheochromocytoma. *Ann Endocrinol (Paris)* 1993;54:337-42.
15. Gerlo EAM, Sevens C. Urinary and plasma catecholamines and urinary catecholamine metabolites in pheochromocytoma: diagnostic value in 19 cases. *Clin Chem* 1994;40:250-6.
16. Hamberger B, Arner S, Eskilsson P, Lindvall N, Werner S, Granberg P-O. Plasma catecholamine levels in the diagnosis and management of pheochromocytoma. *Surg Gynecol Obstet* 1981;152:291-6.
17. Tormey WP, FitzGerald RJ. Lack of uniformity in the clinical approach to the interpretation of urinary catecholamines and their metabolites. *Ir J Med Sci* 1995;164:146-150.
18. Lenders JW, Keiser HR, Goldstein DS, Willemsen JJ, Friberg P, Jacobs MC, Kloppelborg PW, Thien T, Eisenhofer G. Plasma metanephrines in the diagnosis of pheochromocytoma. *Ann Intern Med* 1995;123:101-9.
19. Heron E, Chatellier G, Billaud E, Foos E, Plouin P-F. The urinary metanephrine-to-creatinine ratio for the diagnosis of pheochromocytoma. *Ann Intern Med* 1996;125:300-3.
20. Peaston RT, Lennard TW, Lai LC. Overnight excretion of urinary catecholamines and metabolites in the detection of pheochromocytoma. *J Clin Endocrinol Metab* 1996;81:1378-84.
21. Loh K-C, Shlossberg AH, Abbott EC, Salisbury SR, Tan M-H. Pheochromocytoma: a ten-year survey. *Q J Med* 1997;90:51-60.
22. McNeil AR, Woollard GA. Urinary catecholamines and the diagnosis of pheochromocytoma in Auckland. *N Z Med J* 1997;110:331-3.

23. Lucon AM, Pereira MAA, Mendonca BB, Halpern A, Wajchenbeg BL, Arap S. Pheochromocytoma: Study of 50 cases. *J Urol* 1997;157:1208-12.
24. Eisenhofer G, Lenders JWM, Linehan WM, Walther MM, Goldstein DS, Keiser HR. Plasma normetanephrine and metanephrine for detecting pheochromocytoma in von Hippel-Lindau disease and multiple endocrine neoplasia type 2. *N Engl J Med* 1999;340:1872-9.
25. John H, Ziegler WH, Hauri D, Jaeger P. Pheochromocytomas: can malignant potential be predicted? *Urology* 1999;53:679-83.
26. Goldstein RE, O'Neill JA, Holcomb GW, Morgan WM 111, Neblett WW111, Oates JA, Brown N, Nadeau J, Smith B, Page DL, Abumrad NN, Scott HW. Clinical experience over 48 years with pheochromocytoma. *Ann Surg* 1999;229:755-66.
27. Mediavilla Garcia JD, de la Torre Casares L, Tenorio CH, Fernandez-Torres C, Martinez LA, Mesa FP, de Fuentes MAM. Estudio comparativo de nueve casos con feocromocitoma extraadrenal. *Rev Clin Esp* 1999;199:343-8.
28. Mannelli M, Ianni L, Cilotti A, Conti A. Pheochromocytoma in Italy: a multicentric retrospective study. *Eur J Endocrinol* 1999;141:619-24.
29. Smythe GA, Edwards G, Graham P, Lazarus L. Biochemical diagnosis of pheochromocytoma by simultaneous measurement of urinary excretion of epinephrine and norepinephrine. *Clin Chem* 1992;38:486-92.

Pharmacological tests in phaeochromocytoma diagnosis

There are pharmacological biochemical tests used in the diagnosis of phaeochromocytoma. The glucagon provocation test can be used to stimulate catecholamine release which rises several fold from baseline in phaeochromocytoma. A positive test is where the plasma noradrenaline increases by >3 fold. It is not used when the blood pressure is greater than 160/100 mmHg. False positives do not occur but there is a small false negative rate. The blood pressure response can be blocked using nifedipine without interfering with the catecholamine release that occurs with phaeochromocytomas. The competitive α -adrenergic blocker phentolamine and the vasodilator sodium nitroprusside should be available to guard against the induction of hypertensive crisis. Histamine was used historically for the same purpose but is dangerous. Blood pressure responses were quantified in the era before the development of catecholamine assays. The magnitude of expected pressor responses with the glucagon test is about 105/59 where the basal pressure was normal and 75/45 mmHg when the basal values were elevated¹. The responses occur usually within 2-3 minutes and all are seen within 5 minutes. Neither of these tests has been used in phaeochromocytoma investigation in this or any associated hospital over the past decade. Interestingly, when Eisenhofer et al reported their work on plasma metanephrines in phaeochromocytoma, they found no increase in plasma normetanephrine or metanephrine in 6 phaeochromocytoma patients with positive glucagon stimulations tests. However in one MEN 2 patient who had a glucagon test, no increase in noradrenaline occurred but there was a 10 fold increase in plasma adrenaline and a small (<20%) increase in plasma metanephrine².

The clonidine suppression test has been used to investigate patients with hypertension who have symptoms and signs suggestive of phaeochromocytoma but have moderate increases in plasma or urine catecholamines or metabolites. This test has proved reliable in separating neurogenic hypertension from pheochromocytoma. Clonidine suppresses the sympathetic nervous system by stimulating the α_2 adrenergic receptors in the brain. This reduces plasma noradrenaline by 50% to normal limits in those with neurogenic hypertension but not in those with phaeochromocytoma. False positive or negative tests are rarely encountered³. Clonidine should not be used where there is orthostatic hypotension. Clonidine's vagotonic effect may cause marked hypotension in patients on beta blockers⁴. Theoretically, it may compromise perfusion of the brain and coronary arteries in susceptible individuals. Details are available in Manger and Giffords text⁵.

A protocol follows: Clonidine 33 μ g po is given at time 0. Blood samples are taken at 0, 60, 120 and 180 minutes for noradrenaline and adrenaline. A normal response is where noradrenaline and adrenaline values fall below 500 pg/ml at 120 or 180 minutes after clonidine administration.

Phentolamine Test

This test is now rarely used in clinical practice. It can however be useful when a patient presents acutely in hypertensive crisis as an aid to initial accurate diagnosis. This may be prescient when our patient who died from lactic acidosis is considered⁶.

Phentolamine is given in a 5 mg dose intravenously. Blood pressures are taken every 15 – 20 seconds for 2 minutes and every 30 seconds for the following 10 minutes and continued if there is a positive response. A prompt reduction of 35 mmHg or more systolic and 25 mmHg

diastolic is the cut-off for a positive test. Usually the pressure returns to the higher level within 10 minutes.

False negative tests are often found where there is paroxysmal hypertension in phaeochromocytoma patients. False positive tests are sometimes found in uraemia. Potentially fatal vasomotor collapse has occurred. Side effects such as nasal congestion, lacrimation, pupillary constriction, nausea, sweating, abdominal cramps and diarrhoea have been reported. A modified phentolamine test has been described using the effect of phentolamine in blocking the inhibitory effect of catecholamines on insulin release from the pancreas. In patients with phaeochromocytoma, the insulin levels increase and glucose values fall whereas other patients show little change⁷.

One patient had a request for a phentolamine test.

1. JD is a 29 year old patient with Von Hippel Lindau. He attended a neurologist who arranged for a 24 hr collection when the patients symptoms were consistent with phaeochromocytoma. Urine noradrenaline 162 (<900), adrenaline 485 (increased) (<230), dopamine 1533 (<3300) nmol/24hr. HMMA 15.0 (<40), HVA 25.5 (<40) 5-HIAA 28.5 (<50).

Baseline sample Plasma noradrenaline 1830 pmol/L (450 – 3000), adrenaline 100 (50 – 900), dopamine <30 (100 – 1500).

10 minute sample noradrenaline 1500, adrenaline 80 and dopamine <30.

Pentolinium Test

One patient had the pentolinium test requested according to the Bart's Endocrine Protocols⁸. The patient was D.M., a 57 year old man.

A pituitary tumour was removed in March 1998 by trans-sphenoidal hypophysectomy. He had complained of tiredness, tightness in the head, alopecia and loss of libido. There was also pain in the eyes with reduced visual acuity and blurred vision. This had been progressive for 4 years. MRI showed a pituitary adenoma. The blood pressure was 150/90. Pre-operative cortisol was 1473 nmol/L. TSH 0.37 mIU/L (ref 0.5 – 4.2) FT4 was 7.7 pmol/L (ref 8.0 – 21.0) and he was given l-thyroxine 100ug daily.

Other basal investigations were prolactin 208 mIU/L (ref 50 – 300), FSH 8.2 iu/L, IGF 1 108 ng/ml (ref 108 – 263) Testosterone 0.9 nmol/L (ref 10.3 – 34.5). SHBG 33.0 (ref 10-50 nmol/L). Free testosterone index 2.7 (ref 20 – 350). Post-operatively, hydrocortisone 20 and 10 mg am and pm was given daily. In May 1999, his glucose was 17.7 mmol/L and HBA1c was 6.1%, thus he also had diabetes mellitus.

Insulin stress test in August 1998: the patient was given actrapid insulin 0.2 iu/Kg body weight. Adequate hypoglycaemia on glucometer readings noted.

Time	cortisol	HGH
15 min	221 nmol/L	0.96 ng/ml
30	175	1.02
45	243	1.79
60	390	1.97
90		1.55
120		1.25

Glucose fasting was 4.9 mmol/L, at 30 min 3.0 mmol/L and at 60 min 2.9 mmol/L.

Interpretation. For a normal insulin stress test, blood glucose must be <2.2 mmol/L. Serum cortisol must rise by 170 nmol/L to at least 580 nmol/L. HGH must rise to > 5 ng/ml (40 mU/L)

Water Deprivation Test

Baseline Na 138 mmol/L;	plasma osmolality 309	urine osmolality 716
3 hour sample	plasma osmolality 300 mOsm/Kg	urine osmolality 801
6 hour sample	plasma osmolality 299	urine osmolality 783
7 hour sample	plasma osmolality 291	urine osmolality 781
osmolality units – mOsm/Kg		

These tests show an insufficient anterior pituitary response but the posterior pituitary was conserving water.

Pentolinium is a competitive ganglion blocking drug. Pentolinium 2.5 mg IV is given and plasma catecholamines are taken at 0 and 10 minutes on 9 July 1999. Blood pressure is taken at 0, 10 ,30 and 60 minutes. Readings were at

Time	Blood pressure
0	138/80
10 min	114/78
30	126/69
60	130/81

The failure of pentolinium to suppress catecholamines is characteristic of pheochromocytoma. Normally the catecholamines fall into the normal range. The autonomic responses to intravenous pentolinium may be unpredictable⁹.

However, on request the patient had a collection for urinary catecholamines and the plasma catecholamines were not processed. The values were noradrenaline 503 and 628, adrenaline 36, 35 and dopamine 2409 and 3012. All normal thus the pentolinium test was not necessary.

The patient had a ¹²⁵I MIBG scan on 14 July 1999 and this was negative.

References

1. Roth GM, Flock EV, Kvale WF, Waugh JM, Ogg J. Pharmacologic and chemical tests as an aid in the diagnosis of pheochromocytoma. *Circulation* 1960;21:769-78.
2. Eisenhofer G, Keiser H, Friberg P, Mexey E, Huynh T-T, Hiremagaur B, Ellingson T, Duddempudi S, eijsbouts A, Lenders JWM. Plasma metanephrines are markers of pheochromocytoma produced by catechol-*O*-methyltransferase within tumors. *J Clin Endocrinol Metab* 1998;83:2175-85.
3. Taylor HC, Mayes D, Anton AH. Clonidine suppression test for phaeochromocytoma: Examples of misleading results. *J Clin Endocrinol Metab* 1986;63:238-42.
4. Bravo EL, Gifford RWjr. Pheochromocytoma: diagnosis, localization and management. *N Engl J Med* 1984;311:1298-1303.
5. Manger WM, Gifford RW. Pharmacologic test in the diagnosis of pheochromocytoma in Clinical and experimental pheochromocytoma. 2nd ed. Blackwell Scientific. Cambridge Mass. USA. 1996;226-36.
6. O'Neill G, Tormey WP, Farrell MA, FitzGerald RJ, Finucane J. Phaeochromocytoma and lethal lactic acidemia. *Ir Med J* 1983;86:221.
7. Sergel G, Levy LJ, Chowdhury FR, Rodman HM, Errel NH, Bleicher SJ. A modified phentolamine test for diagnosis of pheochromocytoma. *J Am Med Assoc* 1970;211:266-9.
8. Trainer PJ, Besser M. The Bart's Endocrine Protocols. Churchill Livingstone. Edinburgh. 1995; 50-51.
9. Volle RL, Koelle GB. Ganglionic stimulating and blocking agents. In *The pharmacological basis of therapeutics*. 5thed. Goodman LS, Gilman A. eds. MacMillan, New York, 1975:565-74

Catecholamine secretion and ploidy in pheochromocytoma

Abstract

The aim was to evaluate 24 hr urine catecholamine, HMMA and HVA excretion in relation to ploidy in Pheochromocytoma. Data from 5 diploid and 9 tetraploid tumours showed a significant reduction in urine noradrenaline ($p = 0.0182$) and HMMA ($p = 0.0265$) in diploid tumours but no difference in adrenaline, dopamine and HVA excretion using the Mann-Whitney U test. None of the patients showed recurrence after a mean of 5.7 years of follow-up. A review of published ploidy studies in pheochromocytoma shows that malignancy is more than six times more common in non-diploid tumours but diploidy does not equate with benignity. No biochemical marker is a reliable index of malignancy. All patients should undergo lifetime reviews for recurrence.

Introduction

Although most pheochromocytomas are benign tumours, appropriately treated by surgical excision, recurrent or metastatic disease is reported in 10% to 36% of patients^{1,2,3,4}. Conventional histological features do not predict malignancy in this condition which is defined as regional or distant metastases and/or extensive local invasion. Alternatives such as biochemical indices and nuclear DNA ploidy patterns have been examined as predictors of malignancy. The possible association of elevated dopamine excretion and malignancy was suspected in 1956⁵ and reiterated in the following decade^{6,7}. However, it became apparent that excretion of the dopamine metabolite homovanillic acid (HVA) was variable in malignant pheochromocytoma and may be elevated in some benign tumours. The literature to 1986 was reviewed by Tippet et al⁸.

The largest single study of nuclear DNA ploidy in 184 patients with pheochromocytoma found that approximately one third each were either DNA diploid, tetraploid or aneuploid. Tumour progression occurred in 20% of aneuploid tumours, 15% of tetraploidy and only one case (2%) of the diploid tumours. The conclusion was that DNA diploid tumours did not require the same assiduous follow-up as those with aneuploidy⁹. This study also reported that ploidy was similarly distributed in one third portions in those 55 patients who had preoperative elevations of either plasma dopamine or its urine metabolites but no analyte values were provided. Some of this data was also published in another paper from the same authors but the patients were not double counted in the literature below¹⁰. Other studies found that diploid tumours occurred in as many as 71% of 79 tumours¹¹ and as few as 16% of 19¹². There is some agreement that aneuploidy is associated with a significant risk of tumour

recurrence when compared with diploidy^{9,11,12}. No correlation was found between catecholamine secretion and DNA ploidy patterns but details of the biochemistry were not provided¹¹. A paper (abstract in English) by other authors from the latter Taiwanese institution covering an overlapping time period reported no significant difference in the urinary vanillylmandelic acid excretion between diploid, tetraploid or aneuploid tumours¹³. The data in the latter paper has been disregarded because the time period is completely overlapped in a later paper which stated that DNA ploidy did not correlate with the size of the tumour or the preoperative VMA levels¹⁴. In another study of 17 phaeochromocytomas, patients with a diploid or aneuploid pattern did not differ significantly regarding tissue concentrations of dopa, dopamine, noradrenaline or adrenaline¹⁵. DNA ploidy was not predictive of malignant tumour behaviour in a study of 26 phaeochromocytomas followed for more than five years¹⁶. Details of published ploidy studies in phaeochromocytoma are summarised in Table 1. The number of malignant cases in some of these studies may be an underestimate because of the variable and sometimes unstated lengths of follow-up. The relationship between ploidy and the urinary excretion of catecholamines, HVA and HMMA in our series of phaeochromocytomas is reported.

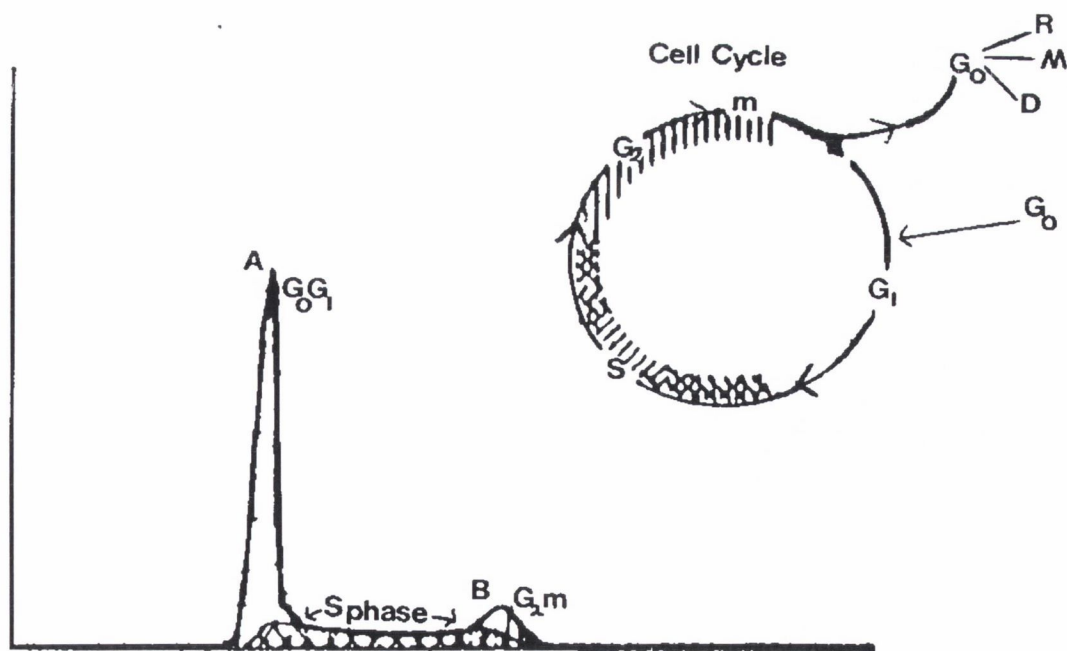
	Table 1				
	N	Diploid	Tetraploid	Aneuploid	Malignant
Lewis PD 20	15	12		3	3 Aneuploid
Klein FA et al 26	1	-	1	-	-
Amberson JB et al 27	19	6	4	9	-
Itakura H et al 28	14	10	-	4	7 Aneuploid
Cope C et al 25	27	8	11	8	2 Tetraploid, 2 Aneuploid
Zhang XH et al 12	19	3	-	16	-
Jung WH et al 29	25	15	8	2	1 Diploid
Nativ O et al 9	184	64	61	59	1 Diploid, 9 Tetraploid, 12 Aneuploid
Gonzales-Campora R et al 30	23	5	9	9	1 Diploid, 1 Tetraploid, 2 Aneuploid
Van Dekken H et al 31	23	6	14	3	-
Pang L-C, Tsao K-C 11	79	56	12	11	5 Tetraploid, 2 Aneuploid
Lai MK et al 14	50	19	21	10	4 Tetraploid
Remmelink M et al 32+A4	16	5	8	3	-
Sasagawa I et al 15	17	9	-	8	-
Heaney AP et al 16	30	13	7	10	3 Diploid, 1 Aneuploid
Tormey WP et al	16	5	11	-	-
Total	558	236 42.3%	167 29.9%	155 28%	6 Diploid, 21 Tetraploid, 29 Aneuploid 10%

Legend:

Ploidy in pheochromocytoma/paraganglioma with details of malignant tumours in the literature listed separately in the right hand column. The totals show the overall ploidy distribution. The distribution of diploidy/aneuploidy/tetraploidy differs significantly between benign and malignant pheochromocytomas ($\chi^2 = 28.79$, 2d.f., $p < 0.0001$).

Methods

DNA ploidy studies were performed on 14 pheochromocytomas where tissue blocks could be retrieved and whose preoperative 24hr urinary free catecholamine excretion values were known. Formalin-fixed paraffin-embedded blocks were used and 50µm sections cut and purely tumour areas were removed for analysis using a modification of the Hedley technique¹⁷. The sections were placed in 10ml glass culture tubes, de-waxed in xylene and re-hydrated through decreasing concentration of ethanol (100%, 95%, 70% and 50%) at room temperature. The tissue was then transferred to centrifuge tubes containing 1ml of pepsin solution. This was incubated at 37°C in a shaking water bath for 30 minutes to 2 hours. The debris was removed leaving a solution of nuclei. Following centrifugation, a 1ml solution of combined fluorochrome propidium iodide / RNAase / Triton X100 was added. The solution was mixed four times and incubated at room temperature for 15 minutes. Clumps of nuclei were broken up by flushing four times through a 21 gage needle and the solution was filtered through a 50µm microcrometer mesh into another tube. Flow cytometric analysis was done within 30 minutes of staining. The cellular DNA content was measured on a fluorescence activated cell sorter flow cytometer (FACSCAN, Becton-Dickinson, Immunocytometry Systems, San Jose, California) using an Argon laser, wavelength 488 nm. The data was collected using LYSIS 11 software and histograms and corresponding cumulative frequencies were printed out and used for the estimation of cell cycle phases G₀, G₁, S, G₂, M.



Legend: Cell cycle and generated histogram. R = resting. M = maturation/differentiation. D = cell death. M = mitoses. S = synthesis-phase.

In our laboratory, the DNA index (DI) range for diploidy is 0.8 - 1.2. The DI for tetraploid population is 1.8 - 2.2 and 20% of cells must fall into this range to exclude the normal 10 - 15% of cells which contain 4N (ie 92 chromosomes) as part of mitosis. An aneuploid peak has a DI outside of these two sets of values and must contain more than 10% of the total cell population to be considered¹⁸. High performance liquid chromatography with electrochemical detection were used for estimating noradrenaline, adrenaline, dopamine, HMMA and HVA¹⁹. The biochemical data from all of the pre-operative urine collections in each patient are included.

Ploidy data from the literature is critically reviewed and appropriate cases are aggregated and listed in Table 1.

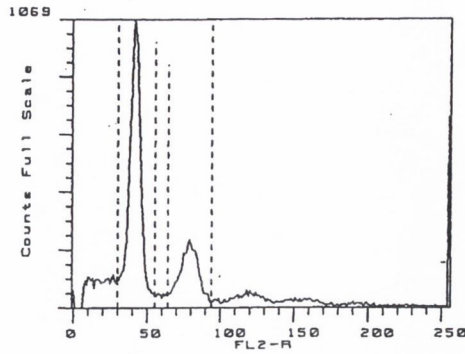
Results

Of the 14 patients studied 5 phaeochromocytomas were diploid and 9 were tetraploid. An example of DNA histogram from one of the patients with a G2M of 28.2% indicating a tetraploid tumour is included below.

One patient died preoperatively and none has evidence of recurrence after a follow-up of 1 to 9 years, average 5.7. There were 19 twenty-four hour urine collections in each group. In the diploid group, one, two, three four and nine collections were done on separate patients and the free catecholamine values were available in all 19, HMMA in 18, and HVA in 9. For the tetraploid group, four collections were done on one patient, three on two patients, two on three and one on three others. Noradrenaline values were available in 16, adrenaline in 15, dopamine in 15, HMMA in 19 and HVA in 13. Using a normal probability plot with the Data Desk 5.0 package, the biochemical data was found to have a non-parametric distribution. Noradrenaline secretion in diploid tumours was significantly lower at 95% confidence level (Mann-Whitney U test, $p = 0.0182$ as was HMMA ($p = 0.0265$). The differences in the other analyte values were not significant; for adrenaline $p = 0.1283$, for dopamine $p = 0.7079$ and for HVA $p = 0.7048$. The results are shown in Figure 1.

DATE: 26-JAN-98

TIME: 17:37:58



Patient Name	: BC 1191/97 PH	Model	: MANL
Case Number	:	Gate	: OFF
Tissue Type	:	Total Events	: 20000
Preparation	: BDIS0001		
Preparation Date	: 26-JAN-98		
Reagents	:		
File Name	: #11:/1/BC001031		
Collection Date	: 26-JAN-98		
Lab	: Becton Dickinson Monoclonal Center		

Region# :	1	2	3	4	5	6	7	8	9	10
-----------	---	---	---	---	---	---	---	---	---	----

Left :	30	30	55	64
Right :	94	55	64	94
Events :	13795	9516	482	3893
% Total :	68.97	47.58	2.41	19.46
% CV :	33.28	10.61	4.84	8.35
Mean :	52	41	60	79
Median :	44	41	59	79
Mode :	41	41	58	79
Mode ct :	1069	1069	55	252
Parent :	1	1	1	1
% Parent:	100.00	68.98	3.49	28.22

FOR RESEARCH PURPOSES ONLY. NOT FOR USE IN
DIAGNOSTIC OR THERAPEUTIC PROCEDURES.

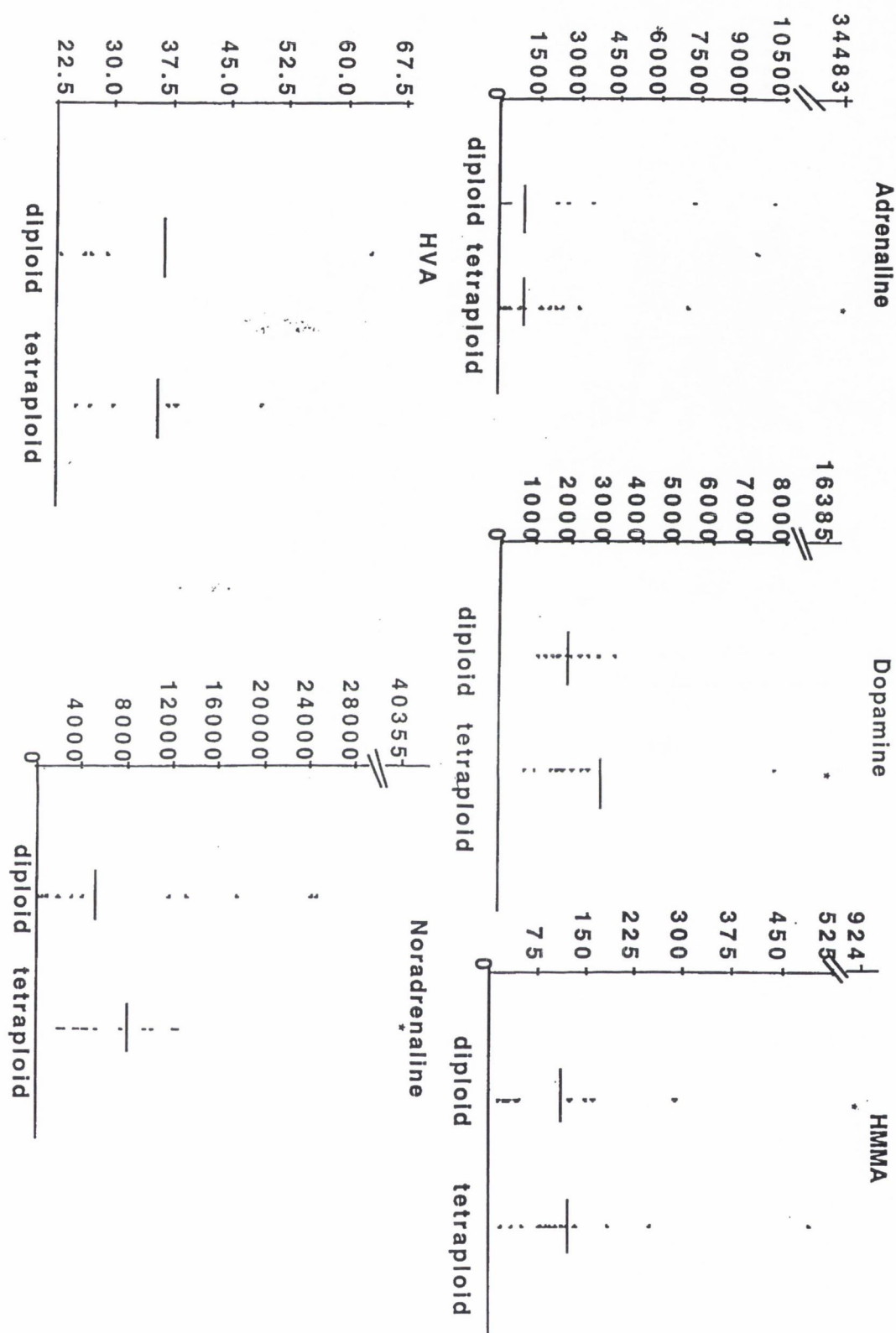


Fig 1 Legend: Urine values for noradrenaline, adrenaline and dopamine in nmol/24hr and HMMA and HVA in μmol/24hr in ploidy groups with median values shown with horizontal bars. Using the Mann Whitney U Test, differences in ploidy groups reached the 95% confidence interval for noradrenaline ($p=0.0182$) and HMMA ($p=0.0265$) only.

Discussion

The lower noradrenaline and HMMA excretion in the diploid group may be related to one patient with an adrenaline secreting tumour who had nine urine collections, six of which were normal for all analytes and none had raised HMMA values. All 13 other tumours were noradrenaline secretors. The lack of a relationship between the excretion of adrenaline, dopamine and HVA and DNA ploidy is not unexpected as the association of an increased dopamine and HVA excretion ultimately proved disappointing as a predictor of malignancy in phaeochromocytoma. From the details shown in Table 1, it is clear that diploidy in phaeochromocytoma does not preclude malignant behaviour although malignancy is more than six times more common in non-diploid tumours. The data from the literature has been included in Table 1 but Lewis²⁰ used a visual classification for analysing the histograms and reported a diploid peak as being diploid even where large numbers of cells had a DNA content greater than a diploid number. Therefore some of those tumours may have been aneuploid by conventional criteria. The ploidy data in Padberg et al²¹ was inadequate for inclusion in Table 1. The report by Proye et al²² was excluded because ploidy was not analysed in any of the benign tumours in their series of 100 phaeochromocytomas. Their selective ploidy study of 18 of 25 malignant phaeochromocytomas only showed that 3 were diploid, 5 aneuploid and 10 tetraploid. The largest study by Nativ et al⁹ covers the period 1960 to 1987 and is an enlargement of a previous study by Hosaka et al²³ from the same institution, published in 1986. The latter data is therefore excluded from Table 1 as it is presumed to be totally included in the extended paper. Both studies had been aggregated in error in a recent commentary on the issue of malignancy in phaeochromocytoma which had rightly concluded that diploidy does not outrule malignant potential²⁴. Malignancy occurred in 10% of all tumours and in 2.5% of diploid tumours in contrast to 12.6% of tetraploid and 18.7% of aneuploid tumours. Chi-squared analysis confirmed that the distribution of diploidy, aneuploidy and tetraploidy differed significantly between benign and malignant tumours. Case duplication was likely in two of the listed papers noted above^{11,14} included in Table 1 as separation of the data is impossible.

Thus, because diploidy does not preclude malignancy, all patients should undergo lifetime review for recurrence. Thus the published evidence contradicts the 1991 recommendation by Cope et al.²⁵, that those with diploid tumours may be spared prolonged intensive follow-up and the similar suggestion by Nativ et al⁹. As details of individual catecholamine excretion and ploidy have not been published in previous papers, a multi-study analysis of this relationship in phaeochromocytoma cannot be done. Ploidy studies do not appear to reflect catecholamine secretion and are not useful in prognosis in individual patients with this condition.

References

1. Drasin H. Treatment of malignant pheochromocytoma. *West J Med* 1978;128:106-11.
2. Van Heerdan JA, Sheps SG, Hamberger B, Sheedy PF, Poston JG, ReMine WH. Pheochromocytoma: Current status and changing trends. *Surgery* 1982;91:367-73.
3. Scott HW Jr, Halter SA. Oncologic aspects of pheochromocytoma: The importance of follow-up. *Surgery* 1984;96:1061-6.
4. Sparagana M. Late recurrence of benign pheochromocytomas: The necessity of long-term follow-up. *J Surg Oncol* 1988;37:140-6.
5. McMillan M. Identification of hydroxytytamine in a chromaffin tumour. *Lancet* 1956;ii:284.
6. Robinson R, Smith P, Whittaker SRF. Secretion of catecholamines in malignant pheochromocytoma. *Br Med J* 1964;1:1422-24.
7. Von Studnitz W. Chemistry and pharmacology of catecholamine secreting tumours. *Pharmacol Rev* 1966;18:645-50.
8. Tippet PA, McEwan AJ, Ackery DM. A re-evaluation of dopamine excretion in pheochromocytoma. *Clin Endocrinol* 1986;25:401-10.
9. Nativ O, Grant CS, Sheps SG, O'Fallon JR, Farrow GM, van Heerdan A, Lieber MM. The clinical significance of nuclear DNA ploidy pattern in 184 patients with pheochromocytoma. *Cancer* 1992;69:2683-7.
10. Nativ O, Grant CS, Sheps SG, O'Fallon JR, Farrow GM, van Heerdan A, Lieber MM. Prognostic profile for patients with pheochromocytoma derived from clinical and pathological factors and DNA ploidy pattern. *J Surg Oncol* 1992;50:258-62.
11. Pang L-C, Tsao K-C. Flow cytometric DNA analysis for the determination of malignant potential in adrenal and extra-adrenal pheochromocytomas and paragangliomas. *Arch Pathol Lab Med* 1993;117:1142-7.

12. Zhang XH, Wei SL, Wang FR, Wang JL, Xie DDW, Liu DW, Zuo LF, Guo JW. Studies on pathological morphology, clinical features and nuclear DNA contents by flow cytometry in adrenal neoplasms. *Chinese Med J* 1992;105:139-43.
13. Huang ST, Lai MK, Sun CC, Chen CS, Chen WH. Pheochromocytoma nuclear pattern analysis by flow cytometry [Chinese]. *Chang Keng i Hsueh - Cheng Gung Med J* 1993;16:188-92.
14. Lai MK, Sun CF, Chen CS, Huang CC, Chu SH, Chuang CK. Deoxyribonucleic acid flow cytometric study in pheochromocytomas and its correlation with clinical parameters. *Urology* 1994;44:185-88.
15. Sasagawa I, Nakada T, Kubota Y, Ishigooka M, Sawamura T, Suzuki Y. Nuclear DNA ploidy pattern and tissue levels of dihydroxyphenylalanine and catecholamines in pheochromocytoma. *Urologia Int* 1995;54:181-3.
16. Heaney AP, O'Rourke D, Arthur K, Beatty OL, Russell CFJ, Sloan JM, Hamilton P, Atkinson AB. Flow cytometric analysis does not reliably differentiate benign from malignant phaeochromocytoma. *Clin Endocrinol* 1996;44:233-238.
17. Hedley DW. Flow cytometry using paraffin-embedded tissue: Five years on. *Cytometry* 1989;10:229-41.
18. Leader MB, Kay EW, Barry Walsh CJ, Dolan J. Assessment of DNA ploidy: a review of methods and significance. *Curr Diag Pathol* 1994;1:105-113.
19. Tormey WP, FitzGerald RF. Phaeochromocytoma - a laboratory experience. *Ir J Med Sci* 1995;164:142-5.
20. Lewis PD. A cytophotometric study of benign and malignant phaeochromocytomas. *Virch Arch* 1971;9:371-6.
21. Padberg BC, Achilles E, Garbe E, Dralle H, Kloppel G, Schroder S. Histology, Immunocytochemistry and DNA-Cytophotometry of adrenal pheochromocytoma (PCC) – a clinicomorphological study of 64 tumours. *Verh Dtsch Ges Path* 1990;74:389-294.

22. Proye C, Vix M, Goropoulos A, Kerlo P, Lecomte-Houcke M. High incidence of malignant pheochromocytoma in a surgical unit. 26 cases out of 100 patients operated from 1971 to 1991. *J Endocrinol Invest* 1992;15:651-63.
23. Hosaka Y, Rainwater LM, Grant CS, Farrow GM, van Heerden JA, Lieber MM. Pheochromocytoma: nuclear deoxyribonucleic acid patterns studied by flow cytometry. *Surgery* 1986;100:1003-8.
24. Patterino F, Bouloux P-M. The diagnosis of malignancy in pheochromocytoma. *Clin Endocrinol* 1996;44:239-41.
25. Cope C, Delbridge L, Philips J, Friedlander M. Prognostic significance of nuclear DNA content in pheochromocytoma. *Aust NZ J Surg* 1991;61:695-98
26. Klein FA, Kay S, Ratliff JE, White FKH, Newsome HH. Flow cytometric determinations of ploidy and proliferation patterns of adrenal neoplasms: an adjunct to histological classification. *J Urol* 1985;134:862-866.
27. Amberson JB, Vaughan ED Jr, Gray GF, Naus GJ. Flow cytometric determination of nuclear DNA content in benign adrenal pheochromocytomas. *Urology* 1987;30:102-4.
28. Itakura H, Kinoshita K, Munakata A, Takamoto S, Minowada S, Aso Y. Flow cytometric analysis of DNA aneuploidy in adrenal tumours. *Jap J Urol* 1992;83:53-8.
29. Jung WH, Yang WI, Park C, Choi IJ. DNA flow cytometry in pheochromocytoma and paraganglioma. *Yonsei Med J* 1992;33:249-57.
30. Gonzales-Camporra R, Diaz Cano S, Lerma-Puertas E, Rios Martin JJ, Salguero Villadiego M, Villar Rodriguez JL, Bibbo M, Davidson HG. Paragangliomas. Static cytometric studies of nuclear DNA patterns. *Cancer* 1993;71:820-4.
31. Van Dekken H, Bosman FT, Teijgeman R, Vissers CJ, Tersteeg TA, Kerstens HMJ, Vooijs GP, Verhofstad AAJ. Identification of numerical aberrations in archival tumours by in situ hybridization to routine paraffin sections: evaluation of 23 pheochromocytomas. *J Pathol* 1993;171:161-71.

32. Remmelink M, Salmon I, Pasteels JL, Ardichvili D, Kiss R. Nuclear DNA content, proliferation index and nuclear size determination in normal and tumoral adrenal tissues, pheochromocytomas and metastases. *Acta Cytologica* 1995;39 (3): 416-22.

Repetition of data in publications on DNA flow cytometry in

Phaeochromocytoma

Ploidy data from eight papers are used in Table 1 of the commentary by Patterino and Bouloux in 1996 on the diagnosis of malignancy in phaeochromocytoma to show the distribution of ploidy in benign and malignant tumours¹. A careful perusal of these demonstrates the difficulties of trying to aggregate data from the literature.

In 1992, Nativ et al published data on ploidy in 184 phaeochromocytomas². This was an extension of a report by Hosaka et al in 1986 on 75 patients from the Mayo Clinic in Rochester, New York³. Further details of the larger study again including ploidy were also separately reported by Nativ et al in 1992⁴. Aggregation of the 1986 and 1992 groups of patients is not valid.

Details of ploidy in phaeochromocytoma from the Chang Gung Memorial Hospital in Taipei, Taiwan were reported in three papers, all of whom were included in the commentary calculations. In 1993, Pang and Tsao detailed 85 consecutive patients with adrenal and extra-adrenal phaeochromocytomas seen between 1979 and 1991⁵. In 1994 Lai et al reported on 58 patients with phaeochromocytoma operated upon at that hospital between September 1980 and April 1993⁶. The time period is stated in the text but not in the summary of these two reports. In 1992 Huang et al reported on 39 patients with phaeochromocytoma treated between 1981 and 1992 and the summary includes the dates and the results⁷. Inclusion of all of these papers in the aggregate analysis would likely involve triple counting in some cases.

On exclusion of the data in Hosaka 1986³, Huang 1992⁷ and Lai 1994⁶ from Table 1 of the commentary by Patterino and Bouloux 1996¹, recalculation of the remaining data shows that the distribution of diploid/aneuploid/tetraploid differs significantly between benign and malignant phaeochromocytomas ($\chi^2 = 18.15$, 2 d.f., $p < 0.0001$). Thus the conclusions in the commentary remain unaltered. These were that until more reliable indicators of malignancy are validated, life-long clinical and biochemical follow-up of phaeochromocytoma remains essential as metastatic spread may occur extremely late in the clinical course⁸.

When aggregating data from the literature, it is unwise to assume that authors comply with the spirit and the letter of the requirements of the International Committee of Medical Journal Editors⁹.

References

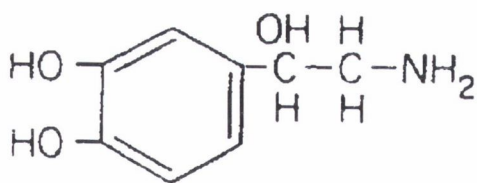
1. Patterino F, Bouloux P-M. (1996) The diagnosis of malignancy in phaeochromocytoma. *Clin Endocrinol* 1996;44: 239-41.
2. Nativ O, Grant CS, Sheps SG, O'Fallon JR, Farrow GM, van Heerden JA, Licber MM. The clinical significance of nuclear DNA ploidy pattern in 184 patients with pheochromocytoma. *Cancer* 1992; 69: 2683-7.

3. Hosaka Y, Rainwater LM, Grant CS, Farrow GM, van Heerdan JA, Lieber MM. Pheochromocytoma: nuclear deoxyribonucleic acid patterns studied by flow cytometry. *Surgery* 1986;100:1003-8.
4. Nativ O, Grant CS, Sheps SG, O'Fallon JR, Farrow GM, van Heerdan JA, Lieber MM. Prognostic profile for patients with pheochromocytoma derived from clinical and pathological factors and DNA ploidy pattern. *J Surg Oncol* 1992;50:258-62.
5. Pang L-C, Tsao K-C. Flow cytometric DNA analysis for the determination of malignant potential in adrenal and extra-adrenal pheochromocytomas or paragangliomas. *Arch Pathol Lab Med* 1993;117:1142-7.
6. Lai M-K, Sun C-F, Chen C-S, Huang C-C, Chu S-H, Chuang C-K. Deoxyribonucleic acid flow cytometric study in pheochromocytomas and its correlation with clinical parameters. *Urology* 1994;44:185-8.
7. Huang S-T, Lai M-K, Sun CC, Chen C-S, Chen W-H. Pheochromocytoma nuclear pattern analysis by flow cytometry. *Chang Gung Med J* 1992;10:188-92.
8. Proye C, Racadout-Leroy N, Vix M, Vermesse B, Vanaille B. Comparative secretory profiles of benign and malignant pheochromocytomas *Ann Chirur* 1994;48:430-4.
9. International Committee of Medical Journal Editors. Uniform requirements for manuscripts submitted to biomedical journals. *New England Journal of Medicine* 1997;336:309-15.

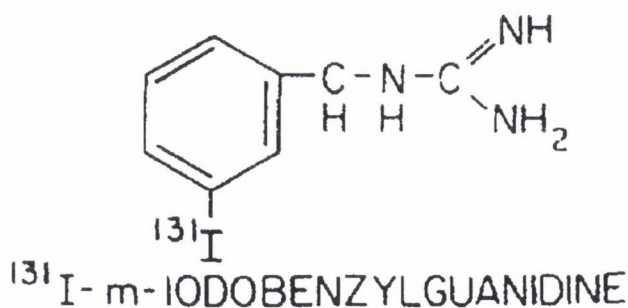
Imaging of paragangliomas and pheochromocytomas.

(A) Radio-isotope imaging

The adrenal gland consists of two embryologically distinct regions. These are also biologically distinct and the cortex was first imaged using radiolabelled cholesterol¹. Catecholamines are synthesised and stored in the adrenal medulla which can be considered as a specialised sympathetic ganglion. The first imaging of the adrenal medulla was achieved using a radiolabelled analog of the adrenergic neuron blocker bretylium². Radiolabelled dopamine and its analogs were also used to image the adrenal medulla³. In the early 1960s, guanethidine was also to act selectively as an adrenergic nerve blocker. Guanidines such as guanethidine and phenethylguanidine share the same transport pathway as noradrenaline in adrenergic nerves and displace noradrenaline from nerve storage granules⁴. The benzyl portion of bretylium and the guanidine group on guanethidine were combined to form the basis for a variety of substituted alkylguanidines with greater antiadrenergic potency⁵. Using cultured bovine adrenomedullary cells as an in-vitro model system, sodium – dependent and independent uptake systems were identified for both noradrenaline and metaiodobenzylguanidine (MIBG). The sodium independent system is a passive diffusion mechanism whereas the classical neuronal uptake-one inhibitors desmethylimipramine and cocaine block the uptake of noradrenaline and MIBG both in vitro and in vivo⁶. Noradrenaline and the alkylguanidine MIBG are sufficiently structurally similar to be recognised by the adrenergic Uptake-1 mechanism and are stored in granules at the adrenergic nerve endings⁷ and are secreted in response to acetylcholine⁸. Unstored noradrenaline is rapidly degraded but the halogenated benzyl ring of MIBG confers resistance to COMT while its guanidino side-chain is resistant to MAO.



NOREPINEPHRINE



¹³¹I- m- IODOBENZYLGUANIDINE

Imaging of the adrenal medulla in the dog was first demonstrated using the bretylium analog ortho¹³¹I iodobenzylmethyl-beta-hydroxyethyl ammonium² in 1979. Imaging of the adrenal medulla in dogs using isomers of ¹³¹I iodobenzylguanidine was found to be clearly superior to labelled dopamine and to iodobenzylmethyl-beta-hydroxyethyl ammonium⁹. The adrenal medullary concentration of the MIBG isomer was 90% higher than the peak adrenocortical uptake of 6-beta ¹³¹I iodo-19-norcholesterol which was widely used to diagnose adrenocortical disease¹⁰. The meta isomer of

iodobenzylguanidine also showed a much lower thyroid radioactivity concentration than the para isomer which gave it a distinct advantage and both showed very little biliary excretion.

¹³¹I-MIBG is normally taken up by the liver, spleen, myocardium and salivary glands. In clinical practice, Lugols iodine 30 mg per day is used routinely to block thyroid uptake of the isotope. The normal adrenal glands are usually not seen but a faint outline is visible in about 16% about 48 to 72 hours after injection. Liver uptake is maximal at 24 hours quickly falling to very low levels by 72 hours. Intraluminal colonic uptake is seen in 15 – 20% of cases and may mimic or obscure tumour activity. Uptake in the spleen, heart and salivary reflect the dense adrenergic innervation of these areas. Cardiac uptake is inversely related to circulating noradrenaline levels and is usually reduced or absent in phaeochromocytoma¹¹. There is little or no in vivo metabolism of MIBG and 90% of an injected dose is recovered in the urine within 4 days. 75-90% is unaltered MIBG and the rest is meta-iodohippuric acid and free iodide¹². Indeed, about 50% of the administered dose of radioactivity appears in the urine within 24 hours.

In a study of 400 cases of suspected phaeochromocytoma from Michigan where there were 35 tumours diagnosed, 441 ¹³¹I-MIBG scans were performed the diagnostic sensitivity was 87.4%. Within these figures, the sensitivity for malignant phaeochromocytoma was 92.4% whereas it was 78.4% in the more common primary sporadic tumours. The specificity was 98.9% but was 100% in familial and malignant cases¹³. These figures are typical as published sensitivities range from 79% to 89% and specificities 94% to 99%^{14-18,26}. A later paper from Michigan reported that “¹³¹I-MIBG scintigraphy localised the tumor accurately in 82.3% (28/34) of patients. In 4 other patients (11.8%), the scan was partly accurate. Three were interpreted as adrenal gland uptake, but operation revealed extra-adrenal tumours in para-aortic, para-renal, and retrocaval locations”¹⁹. Details are shown in Table 1.

Table 1

	Sensitivity	Specificity
Michigan ¹³	87.4	99
Southampton ¹⁴	89	95
Mayo Clinic ¹⁶	79	96
Tours ¹⁷	89	94
(Combined German experience) ¹⁵	88	99
Brazil ¹⁸	88	--
France ²⁶	91	96

Legend: Sensitivities and specificities of ¹³¹I MIBG in the diagnosis of phaeochromocytoma.

Uses of ^{123}I MIBG

When used to locate phaeochromocytomas, ^{131}I MIBG has a false positive rate of about 13%. When compared to ^{131}I , the isotope ^{123}I has superior dosimetry, a better detection efficiency and spatial resolution. When diagnostic doses of 18.5 MBq of ^{131}I MIBG and 370 MBq ^{123}I MIBG, which have nearly equivalent dosimetries were compared, the latter gave better images and in 8 of 18 patients showed lesions that were not shown using the ^{131}I marker. ^{123}I has the further advantage of permitting single photon computed tomography (SPECT)²⁰. However ^{131}I is less costly and has a two week shelf life. Gamma cameras have a low detection efficiency for the 364 keV photon of ^{131}I whereas the 159 keV photon of ^{123}I is efficiently detected. Myocardial uptake with ^{123}I MIBG is comparable to ^{201}Tl and the normal adrenal medulla, lacrimal glands and uterus may usually be seen. A direct correlation has been shown between the percentage of the injected dose taken up by the tumour and the number of neurosecretory granules seen on electronmicroscopy²¹. Impaired uptake and rapid clearance of the ^{123}I isotope from the heart and liver is also a feature in phaeochromocytomas and in autonomic neuropathies. Cost and a limited shelf life are disadvantages with the ^{123}I isotope.

^{123}I MIBG is considered the ideal agent for imaging of phaeochromocytomas and a sensitivity and specificity of 93% and 100% have been reported²². A comparative study from Tokyo of ^{123}I compared to ^{131}I label in MIBG scanning for phaeochromocytoma found that the diagnostic sensitivities were 90% and 85.7% respectively with both having 100% specificity. The image quality with the ^{123}I label was better. Also, the normal adrenal was visualized in 50% of the ^{123}I scans. There were however only 14 patients in the ^{131}I group and 8 in the ^{123}I group who had phaeochromocytomas²³. The adrenal medulla is seen even when there is an extramedullary phaeochromocytoma using the ^{123}I label. MIBG is excreted in the urine and therefore uptake from normal bladder excretion may obscure lesions in the pelvic area. Thus the bladder must be emptied before commenting on pelvic images. Rare cases of false positive ^{123}I MIBG scans have been reported especially where the tracer is retained in the renal pelvis²⁴. Other labeled radiopharmaceuticals such as $^{99\text{m}}\text{Tc}$ DTPA have been used to assess the relationship of abnormal MIBG uptake to liver, heart and bone.

A Danish study of 30 patients evaluating ^{123}I MIBG SPECT in the location of phaeochromocytoma reported a diagnostic sensitivity of 100%, specificity 95% a positive predictive value of 95% and a negative predictive value of 100%²⁵.

A wide range of neuroendocrine tumours may show uptake of MIBG. These include neuroblastoma, medullary carcinoma of the thyroid, carcinoids, non-functioning paragangliomas, bronchial carcinomas, Merkel-cell skin cancers and neuroendocrine granule containing schwannomas. ^{111}In labelled octreotide may also be used in scanning for phaeochromocytoma but its role may be no more than complimentary²⁶. A small Dublin study found that labeled octreotide could identify tumours that were negative with MIBG²⁷. When compared to ^{123}I MIBG, screening with $^{99\text{m}}\text{Tc}$ -DMSA is quite

insensitive. It identified only 1 of 7 sites of deposits of malignant pheochromocytoma which had been earlier identified by MIBG²⁸.

The uptake of MIBG is inhibited both in vitro and in vivo by tricyclic antidepressants, verapamil, labetalol, cocaine, amphetamines, reserpine, some neuroleptics and phenylpropanolamine. The introduction of calcium channel blockers after MIBG injection may prolong the isotope half-life in targeted tissues²⁹. Other α - and β -blockers do not affect MIBG uptake and should not be discontinued prior to scanning. An extensive list of drugs which may potentially affect MIBG uptake has been published. Interestingly, the effect of calcium channel blockers is listed as uncertain³⁰.

False positive MIBG scans are rare³¹. A recent study of 104 patients with pheochromocytoma and paraganglioma ¹³¹MIBG was negative in 35.5% of tumours <20 cm³ and in 2.6% of those >20 cm³. The results of scanning significantly correlated with the tumour volume and the largest diameter. It was concluded that tumour size is the only predictive factor for the outcome of the scintiscan³².

In the clinical investigation of these tumours, localisation is attempted using a number of imaging modalities. There is no general agreement to date on which mode is the most appropriate in different contexts and clinical practice varies. Local availability of radiology equipment is an important factor. Gamma probes have been used in the intraoperative localisation of neuroblastoma in children using ¹²³I or ¹²⁵I radiolabeled MIBG³³.

(B) CT and MRI

Adrenal masses of varying pathology are found in 0.35 – 4.4% of patients imaged with CT for reasons other than suspected adrenal pathology. The great majority are adrenal adenomas. These adrenal “incidentalomas” have also been reported in 1.4% to 5.7% of large autopsy series³⁴. Pheochromocytomas must be distinguished from adrenal cysts, myelolipomas, carcinoma and metastases, and surgically excised. The imaging appearances are particularly important in the 10% of pheochromocytomas that may be biochemically silent.

On CT, pheochromocytomas are usually discrete rounded or oval masses of an average 5cm diameter with similar density to liver. Central necrosis is frequent and may be extensive enough to cause cystic change. Usually cases associated with MEN 2 or Von Hippel-Lindau are smaller, probably because they are actively sought in family studies rather than otherwise found.

Most pheochromocytomas enhance markedly on CT after contrast injection. Ionic contrast medium may cause the release of a surge of catecholamines which may be dangerous in untreated patients. Non-ionic contrast medium does not seem to cause such problems³⁵. On MRI, most pheochromocytomas are hypointense on T1-weighted images and very hyperintense on T2-weighted images. However these appearances are not specific and there is overlap with oedematous and necrotic adrenal masses³⁵.

In 1985, a multicenter study of 99 patients, Chatal and Charbonnel reported that CT shows 95% of intra-adrenal phaeochromocytomas and that there was 80% agreement between CT and MIBG³⁶. In a 1993 relatively small comparative study of computerised tomography (CT), magnetic resonance imaging (MRI) and ¹³¹I MIBG in the pre- and postoperative evaluation of 36 patients with suspected paraganglioma, CT and MRI gave 100% and MIBG 82% sensitivity whereas the specificities for CT and MRI were 66% with 100% for MIBG³⁷. In a study of 50 cases, Lucon et al¹⁸ reported a sensitivity of 94% for CT and 100% for MRI. In an editorial on the role of these imaging modalities, Chatal³⁸ concludes CT and MRI are the most suitable techniques for the accurate localisation of intra-adrenal tumours. Were it not necessary to check for extra-adrenal sites, CT alone would be sufficient but MIBG scans should be done first since it provides whole body information especially regarding extra-adrenal paragangliomas and metastases. MRI is complementary to MIBG in the exploration of the abdomen and pelvis. Continuous rotation and/or spiral CT provide spatial resolution superior to MRI and are more effective in localising thoracic tumours of less than 5 mm^{39,40}.

To try to improve the diagnostic accuracy from MRI scans, many authors noted that phaeochromocytomas consistently displayed higher signal intensity than benign adenomas and malignant lesions on T2-weighted imaging⁴¹⁻⁴³. However, a recent study has demonstrated that there is considerable overlap between the MRI appearance of phaeochromocytoma and other adrenal lesions and concluded that phaeochromocytoma cannot be excluded on the basis of a lack of high signal intensity on T2-weighted imaging⁴⁴. The sensitivity of MRI in diagnosing phaeochromocytoma was 64.7%, specificity 88%, positive predictive value 64.7% and negative predictive value of 88%. Their conclusion was that "indiscriminate MR screening of hypertensive patients with suspected phaeochromocytoma without appropriate biochemical screening is not advised".

Despite the evidence, it is still stated by some that MRI is the method of choice when adrenal masses cannot be differentiated by CT⁴⁵. MRI is optimal for the pre-operative anatomical delineation of the lesion.

(C) Abdominal Ultrasound

This is a convenient, cheap and commonly used technique. There is a broad spectrum of abdominal sonographic appearances with phaeochromocytoma⁴⁶. It may show tumours greater than 2 cm and also cystic change. Published sensitivities have varied from 89% to 93%^{18,47}. In an abdominal ultrasound study of 1500 hypertensive patients, only one phaeochromocytoma was found among 8 'incidentalomas' of the adrenal⁴⁸. Ultrasound is particularly useful and convenient for the investigation of abdominal masses in children⁴⁹.

(D) Intravenous Urography (Pyelography), Renography and Angiography

None of these investigations is necessary in phaeochromocytoma, but may form part of the investigation of secondary hypertension. In an analysis of 121 patients with primary

adrenal tumours, the diagnostic sensitivities of IVP and ultrasound were 59% and 71% respectively⁵⁰. In the 1970s, IVP and nephrotomography was reported to localise up to 70% of adrenal tumours^{51,52}. Small tumours less than 2.5 cm in diameter were not shown⁵³. Angiography is only used in particular anatomical circumstances pre-surgery. The lack of a structured approach to the use of imaging modalities in our patients has been reported⁵⁴.

(E) Positron Emission Tomography

Positron emission tomography (PET) using 2-¹⁸F fluoro-2-deoxy-D-glucose (FDG) has been used in phaeochromocytoma to image the tumour. Tumour uptake was detected in 22 of 29 patients; 7 of 12 benign and 15 of 17 malignant phaeochromocytomas and their metastases concentrated the FDG. In four patients with negative MIBG scans, the FDG uptake was intense. In the 16 tumours that concentrated MIBG and FDG, MIBG images were judged superior in 56% and equal or better in 88%. Uptake was found in a higher percentage of malignant than benign phaeochromocytomas. The conclusion was that FDG PET is especially useful where MIBG is negative⁵⁵.

References

1. Lieberman LM, Beierwaltes WH, Conn JW, Ansari AN, Nishiyama H. Diagnosis of adrenal disease by visualization of human adrenal glands with ¹³¹I 19-iodocholesterol. *N Engl J Med* 1971;285:1387-93.
2. Wieland DM, Swanson DP, Brown LE et al. Imaging the adrenal glands with an I-31-labelled antiadrenergic agent. *J Nucl Med* 1979;20:155-58.
3. Ice RD, Wieland DM, Beierwaltes WH, Lawton RG, Redmond MJ. Concentration of dopamine analogs in the adrenal medulla. *J Nucl Med* 1975;16:1147-51.
4. Nickerson M, Collier B. Drugs inhibiting adrenergic nerves and structures innervated by them. In *The Pharmacological Basis of Therapeutics*, 5th ed, Goodman LS, Gillman A, eds., New York, Macmillan, 1975,553-64.
5. Short JH, Darby TD. Sympathetic nervous system blocking agents. 111. Derivatives of benzylguanethidine. *J Med Chem* 1967;10:833-40.
6. Tobes MC, Jaques S, Wieland DM, Sisson JC. Effect of Uptake-one inhibitors on the uptake of norepinephrine and metaiodobenzylguanidine. *J Nucl Med* 1985;26:897-907.
7. Gasnier B, Rosin MP, Scherman D, Coomaert S, Desplanches G, Henry JP. Uptake of meta-iodobenzylguanidine by bovine chromaffin granule membranes. *Molec Pharmacol* 1986;29:275-80.

8. Jaques SJr, Tobes MC. Comparison of the secretory mechanisms of metaiodobenzylguanidine (MIBG) and norepinephrine (NE) from cultured bovine adrenomedullary cells. *J Nucl Med* 1985;26:17
9. Wieland DM, Wu J-L, Brown LE, Mangner TJ, Swanson DP, Beierwaites WH. Radiolabelled adrenergic neuron-blocking agents: Adrenomedullary imaging with (¹³¹I)Iodobenzylguanidine. *J Nucl Med* 1980;21:349-53.
10. Thrall JH, Freitas JE, Beierwaltes WH. Adrenal scintigraphy. *Semin Nucl Med* 1978;7:23-41.
11. Nakajo M, Shimabukuro K, Miyaji N, Shimada J, Shirono K, Sakata H, Yoshimura H, Yonekura R, Shinohara S. Rapid clearance of iodine-131 MIBG from the heart and liver patients with adrenergic dysfunction and phaeochromocytoma. *J Nucl Med* 1985;26:357-65.
12. Mangner TJ, Tobes MC, Wieland DW, Sisson JC, Shapiro B. Metabolism of iodine-131 metaiodobenzylguanidine in patients with metastatic pheochromocytoma. *J Nucl Med* 1986;27:37-44.
13. Shapiro B, Copp JE, Sisson JC, Eyre PL, Wallis J, Beierwaltes WH. Iodine-131 metaiodobenzylguanidine for the locating of suspected pheochromocytoma: experience in 400 cases. *J Nucl Med* 1985;26:576-85.
14. Ackery DM, Tippet PA, Condon BR, Sutton HE, Wyeth P. New approach to the localization of phaeochromocytoma: imaging with iodine 131-meta-iodobenzylguanidine. *Br Med J* 1984;288:1587-91.
15. Editorial. Clinical value of adrenomedullary scintigraphy with 131I-MIBG. *Nucl Comp* 1983;14:1587-91.
16. Brown ML, Sheps SG, Sizemore G. MIBG in the evaluation of suspected pheochromocytoma: Mayo Clinic experience. *J Nucl Med* 1984;25:111 (abstr).
17. Baulieu JL, Guillotauu C, Canbon C et al. MIBG scintigraphy: a one-year experience. *J Nucl Med* 1984;25:111(abstr).
18. Lucon AM, Pereira MAA, Mendonca BB, Halpern A, Wajchenbeg BL, Arap S. Pheochromocytoma: study of 50 cases. *J Urol* 1997;157:1208-12.
19. Cheung PSY, Thompson NW, Dmuchowski CF, Sisson JC. Spectrum of pheochromocytoma in the ¹³¹I-MIBG era. *World J Surg* 1988;12:546-51.
20. Lynn MD, Shapiro B, Sisson JC, Beierwaltes WH, Meyers LJ, Ackerman R, Mangner TJ. Pheochromocytoma and the normal adrenal medulla: improved visualisation with I-123 MIBG scintigraphy. *Radiology* 1985;155:789-92.

21. Bomanji J, Levison DA, Flatman WD, Horne T, Bouloux PM, Ross G, Britton KE, Besser GM. Uptake of iodine-123 metaiodobenzylguanidine by pheochromocytomas, paragangliomas and neuroblastomas: a histopathological comparison. *J Nucl Med* 1987;28:973-8.
22. Bomanji J, Conry BG, Britton KE, Reznick RH. Imaging neural crest tumours with I-123 metaiodobenzylguanidine and X-ray computed tomography: a comparative study. *Clin Radiol* 1988;39:502-6.
23. Furuta N, Kiyota H, Yoshigoe F, Hasegawa N, Ohishi Y. Diagnosis of pheochromocytoma using [123]-compared with [131]-metaiodobenzylguanidine scintigraphy. *Int J Urol* 1999;6:119-124.
24. Bathmann J, Neumann HP, Sigmund G, Moser E. False-positive diagnosis of a pheochromocytoma with I-123 metaiodobenzylguanidine. *Clin Nucl Med* 1994;19:221-3.
25. Nielsen JT, Nielsen BV, Rehling M. Location of adrenal medullary pheochromocytoma by I-123 metaiodobenzylguanidine SPECT. *Clin Nucl Med* 1996;21:695-9.
26. Kopf D, Bockisch A, Steinert H, Hahn K, Beyer J, Neumann HP, Hensen J, Lehnert H. Octreotide scintigraphy and catecholamine response to an octreotide challenge in malignant pheochromocytoma. *Clin Endocrinol* 1997;46:39-44.
27. Li Voom Chong SW, Darby C, Freyne P, Cullen MJ. The use of metaiodobenzylguanidine (MIBG) compared to radiolabelled octreotide scans in the localisation of suspected pheochromocytoma. *Ir J Med Sci* 1998;167,Suppl 3:5.
28. Limouris GS, Giannakopoulos V, Stavrika A, Toubanakis N, Vlahos L. Comparison of In-111 pentetreotide, Tc-99m (V)DMSA and I-123 mIBG scintimaging in neural crest tumors. *Anticancer Res* 1997;17:1589-92.
29. Manger WM, Gifford RW. Isotope localization of adrenal pheochromocytoma. In *Clinical and Experimental pheochromocytoma*. 2nd ed, Cambridge Mass, USA. Blackwell Scientific, 313-314.
30. The adrenal gland. In. *Clinical Nuclear Medicine*, 2nd Ed. Eds MN Maisey, KE Britton, DL Gilday, Chapman Hall Medical. London 1991;281-91.
31. Letizia C, De Toma G, Massa R, Corsi A, Caliumi C, Subioli S, d'Erasmus E. False-positive diagnosis of adrenal pheochromocytoma on iodine-123-MIBG scan. *J Endocrinol Invest* 1998;21:779-83.

32. Nguyen HH, Proye CA, Carnaille B, Combemamle F, Pattou FN, Huglo D. Tumour size: the only predictive factor for ¹³¹I MIBG uptake in pheochromocytoma and paraganglioma. *Aust NZ J Surg* 1999;69:350-3.
33. Martelli H, Ricard M, Larroquet M, Wioland M, Paraf F, Faber M, Josset P, Helardot PG, Gauthier F, Terrier-Lacombe MJ, Michon J, Hartmann O, Tabone MD, Patte C, Lumbroso T, Gruner M. Intraoperative localization of neuroblastoma in children with ¹²³I- or ¹²⁵I-radiolabelled metaiodobenzylguanidine. *Surgery* 1998;123:51-57.
34. Peppercorn PD, Grossman AB, Reznick RH. Imaging of incidentally discovered adrenal masses. *Clin Endocrinol* 1998;48:379-88.
35. Mukherjee JJ, Peppercorn PD, Reznick RH, Patel V, Kaltas G, Besser GM, Grossman AB. Pheochromocytoma: effect of non-ionic contrast medium in CT on circulating catecholamine levels. *Radiology* 1997;202:227-31.
36. Chatal JF, Charbonnel B. Comparison of iodobenzylguanidine imaging with computed tomography in locating pheochromocytoma. *J Clin Endocrinol Metab* 1985;61:769-72.
37. Maurea S, Cuocolo A, Reynolds JC, Tumei AA, Begley MG, Linehan WM, Norton JA, Walther MM, Keiser HR, Neumann RD. Iodine-131-metaiodobenzylguanidinescintigraphy in preoperative and postoperative evaluation of paragangliomas: comparison with CT and MRI. *J Nucl Med* 1993;34:173-9.
38. Chatal J-F. Can we agree on the best imaging procedure(s) for localization of pheochromocytomas? *J Nucl Med* 1993;34:180-1.
39. Davis SD. CT evaluation for pulmonary metastases in patients with extrathoracic malignancy. *Radiology* 1991;180:1-12.
40. Webb WR, Sostman HD. MR imaging of thoracic disease: clinical uses. *Radiology* 1992;182:621-30.
41. Glazer GM, Woolsey EJ, Borrello J, Francis IR, Aisen AM, Bookstein F, Amendola MA, Gross MD, Bree RL, Martel W. Adrenal tissue characterization using MR imaging. *Radiology* 1986;158:73-9.
42. Reinig JW, Doppman JL, Dwyer AJ, Johnson AR, Knop RH. Adrenal masses differentiated by MR. *Radiology* 1986;158:81-4.
43. Lee MJ, Hahn PF, Papanicolaou N, Egglin TK, Saini S, Mueller PR, Simeone JF. Benign and malignant adrenal masses: CT distinction with attenuation coefficients, size and observer analysis. *Radiology* 1991;179:415-8.

44. Varghese JC, Hahn PF, Papanicolaou N, Mayo-Smith WW, GAA JA, Lee MJ. MR differentiation of pheochromocytoma from other adrenal lesions based on qualitative analysis of T2 relaxation times. *Clin Radiol* 1997;52:603-6.
45. Neumann K, Langer R. Imaging methods in diagnosis of pheochromocytoma. *Zentralbl Chir* 1997;122:438-42.
46. Schwerk WB, Gorg C, Gorg K, Restrepo IK. Adrenal pheochromocytomas: a broad spectrum of sonographic presentation. *J Ultrasound Med* 1994;13:517-21.
47. Kluglich M, Duelli R, Zoller WG, Middeke M. Ultrasound of incidental tumors of the adrenal gland and endocrine hypertension. *Bildgebung* 1993;60:144-6.
48. Feltynowski T, Januszewicz W, Chodakowska J, Wocial B, Pruszczyk P, Kakubowski W, Leowska E, Pacho R, Szamowska R. Evaluation of the usefulness of certain localization methods in diagnosis of pheochromocytoma. *Pol Tyg Lek* 1991;46:639-40.
49. Muller MF, Krestin GP, Willi UV. Abdominal tumours in children. A comparison between magnetic resonance tomography (MRT) and ultrasonography. *Rofo Fortschr Geb Rontgenstr Neuen Bildgeb Verfahr* 1993;158:9-14.
50. Schwarz RJ, Schmidt N. Efficient management of adrenal tumors. *Am J Surg* 1991;161:576-9.
51. Pickering R, Hartman G, Weeks R, Sheps SG, Hattery RR. Excretory urographic localisation of adrenal cortical tumors and pheochromocytoma. *Radiology* 1975;111:345-9.
52. Zelch J, Meaney T, Belhdbek G. Radiologic approach to the patient with suspected pheochromocytoma. *Radiology* 1974;111:279-84.
53. Remine WH, Chong GC, van Heerden JA, Sheps SG, Harrison EG Jr. Current management of phaeochromocytoma. *Ann Surg* 1974;179:740-8.
54. Tormey WP, FitzGerald RJ. Lack of uniformity in the clinical approach to the interpretation of urinary catecholamines and their metabolites. *Ir J Med Sci* 1995;164:146-50.
55. Shulkin BL, Thompson NW, Shapiro B, Francis IR, Sisson JC. Pheochromocytomas: imaging with 2-[fluorine-18]fluoro-2-deoxy-D-glucose PET. *Radiology* 1999;212:35-41.

¹²³I-metaiodobenzylguanidine scans in phaeochromocytoma diagnosis: current clinical practice and relationship to biochemical screening.

Summary

The use in practice of ¹²³I-metaiodobenzylguanidine (MIBG) scans in phaeochromocytoma diagnosis in our teaching hospital over an eight year period is reported. MIBG scans were performed in 43 patients and the clinical context is reviewed and reported in 42 of these. A paraganglioma and 5 phaeochromocytomas were found in the MIBG series. There were no false negative MIBG scans. CT was performed in 61.9%, abdominal ultrasound in 45.3% and MRI in 7.1% of the patients. Urinary catecholamines and metanephrines are considered the best screening tests for phaeochromocytoma and false positive biochemistry values were associated with MIBG scans in 35% of the cases. Despite published limitations on its sensitivity, MIBG scans were the only modality used in 21.4%. 25% of the MIBG scans were performed for clinical reasons when the biochemical screen was within the reference range. MIBG is superior to CT and MRI for the localisation of suspected recurrence after surgery and is also useful for the detection of extra adrenal tumours and metastases.

Introduction

Use of evidence-based protocols is the most efficient method of addressing uncertainty in diagnosis. Protocols for the investigation of catecholamine secreting tumours have been published^{1,2}. These are a compromise between what is available and affordable, and what is possible. In searching for possible phaeochromocytomas, we previously recommended that ultrasound and CT scanning should follow a positive screen for urinary catecholamines or should be performed where the biochemistry is negative yet clinical suspicion remains strong^{1,2}. MIBG scans were recommended if CT is positive but urinary catecholamine values are equivocal or if CT is negative despite high biochemical values. MRI was suggested where CT and MIBG were negative yet clinical suspicion remained strong and urine biochemistry was positive. Others have recommended that ¹³¹I-MIBG should be the first localisation procedure performed as conventional CT gives no additional information when a tumour has been localised by MIBG³. However a 10% false negative rate has been reported with scintigraphy in phaeochromocytoma especially for intra-adrenal cases and CT has been found to be more reliable for adrenal tumours⁴. The specificity of MIBG scintigraphy has been recorded at 100% for both adrenal and extra-adrenal tumours⁵. An earlier series reported a specificity at 98.9% with a sensitivity at 87.4%⁶. In hypertensive patients, the triad of sweating attacks, tachycardia and headaches is reported to have a specificity of 93.8% and a sensitivity of 90.9% for phaeochromocytoma while absence of these symptoms outrules the disease in 99.9%⁷. Thus the clinical history is central to any protocol for evaluating patients with suspected phaeochromocytoma.

In phaeochromocytoma about 50% of patients have sustained hypertension and about 10% of tumours are malignant. With extra-adrenal phaeochromocytomas / paragangliomas, about 50% of those in the retroperitoneal space are more likely to be malignant of which less than half are catecholamine secretors. About 90% of non-functional paragangliomas are detected by MIBG scans⁸. The frequency of malignancy is related to the anatomical site⁹. As well as from malignancy, death may also supervene from the acute consequences of catecholamine toxicity, such as acute myocardial infarction or lactic acidosis¹⁰. Therefore, tumour detection

is crucial. MIBG is an analog of the adrenergic neuron blocker guanethidine. It shares the same uptake, storage and release mechanisms as noradrenaline in the sympathetic nerve endings^{11, 12}.

This chapter reports on the actual use of MIBG scans in clinical practice in the investigation of phaeochromocytoma, the relationship with biochemical evaluation and the relationship with recommended practices.

Methods

From 1990 – 1997, 43 patients had ¹²³I-MIBG scans in the investigation of phaeochromocytoma/paraganglioma. One patient was scanned twice. Clinical records from 42 patients were retrieved to establish the clinical context for the investigations performed in each case. The drug therapy of each patient was noted as a potential cause of interference in the biochemical analysis and in the noradrenaline uptake and storage mechanisms used by MIBG. Lugol’s iodine 30 mg per day was given orally on the day prior to injection of MIBG and for the succeeding seven days. ¹²³I-MIBG with a specific activity of 100 MBq was administered intravenously by slow injection over about 20 sec in a buffered bacteriostatic saline. Images were obtained using a large-field-of-view gamma camera with a high-energy, parallel-hole collimator linked to a dedicated computer. Scintiscans were performed one to two days after injection. Urine collections for noradrenaline, adrenaline and dopamine had been performed in all but one patient and 4-hydroxy-3-methoxy mandelic acid (HMMA) and homovanillic acid (HVA) was performed in all but three. As detailed in chapter 4, urinary free catecholamines and HMMA and HVA were measured by high performance liquid chromatography with electrochemical detection in 24 hr acidified collections¹³ and the reference ranges used had been determined from hypertensive patients by Davidson and FitzPatrick whose method we had adapted¹⁴. The laboratory provides a catecholamine assay service for seven hospitals outside its catchment area and also serves local primary care facilities.

Results

Clinical notes from 42 of 43 patients were retrievable. The notes from the remaining patient, who was referred from another hospital for scintiscanning, could not be located. The male/female ratio was 1.8/1 and the ages ranged from 14 to 76 yr with a median of 54. As well as MIBG scans, CT was performed in 25 (59.5%), abdominal ultrasound in 19 (45.2%), MRI in 3 (7.1%), IVP in 6 (14.3%), MAG 3 renograms in 4 (9.5%), and two had bone scans. MIBG scans were the only imaging modality used in 9 (21.4%). Diagnoses additional to arterial hypertension or phaeochromocytoma/paraganglioma were made in 24 (57.1%) patients. These are listed in Table 1. None of those with hyperparathyroidism or medullary carcinoma of the thyroid had Multiple Endocrine Neoplasia.

Table 1

- Adrenocortical adenoma x 2
- Adrenal benign vascular cyst
- Renal artery stenosis x 2
- Hypertensive renal failure
- Membranoproliferative glomerulonephritis
- Medullary carcinoma of the thyroid and parkinsonism

Thymoma
Ganglioglioma of the spinal cord
Lymphocoele
Acoustic Schwannoma
Mucinous adenocarcinoma of uncertain origin
Hyperparathyroidism x 2
Sarcoidosis and hyperthyroidism
Temporal lobe epilepsy x 2
Psittacosis
Chronic urticaria
Basal ganglia haemorrhage and arteriovenous malformation
Alcoholism and oesophagitis.
Depression x 2.

The above diagnoses were made in addition to arterial hypertension or phaeochromocytoma/paraganglioma in 24 of 42 patients studied in detail.

In this study, the MIBG scans were positive in the one paraganglioma and in all five phaeochromocytomas where used and there were no false negatives. The paraganglioma was metastatic and presented with Cushing's syndrome from ectopic ACTH secretion and another patient had cerebral and spinal metastases from a malignant adrenal phaeochromocytoma. For CT, the sensitivity and specificity in phaeochromocytoma diagnosis was 100% and 75% respectively because there were 5 other adrenal masses (false positives) – metastatic carcinoma, benign vascular adrenal cyst, lymphocoele, renal cyst and an adrenal adenoma. With 2 false positives and 15 true negatives, the specificity for ultrasound was 88%. Ultrasound was positive in one of the two phaeochromocytomas where used.

No raised biochemical parameter was found in 11 (25.6%) but in 15 (34.9%) cases, false positive elevations in one or more of the urine biochemistry values were recorded. These values are shown in Figure 1 as multiples of the upper limit of normal (ULN). Drug interference with the biochemical assays was possible in eight cases (19%) and the agents were alpha methyl dopa (aldomet), L-dopa, paracetamol, paroxetine once each and labetalol five times. There were 23 (54.8%) patients with symptoms compatible with phaeochromocytoma but only two had the classic triad of paroxysms of sweating, tachycardia and headaches and these cases proved positive. Other associated clinical symptoms included flushing, anxiety, panic, palpitations, and dizziness. All except two patients were hypertensive and paroxysmal surges were present in 14 (34.1%). Two patients who had phaeochromocytoma previously removed had scans to investigate possible recurrence. Of drugs that interfere with MIBG uptake, five patients were taking labetalol and another took prochlorperazine. Tumour was outruled in these cases by the performance of CT scans and repeated biochemical analysis.

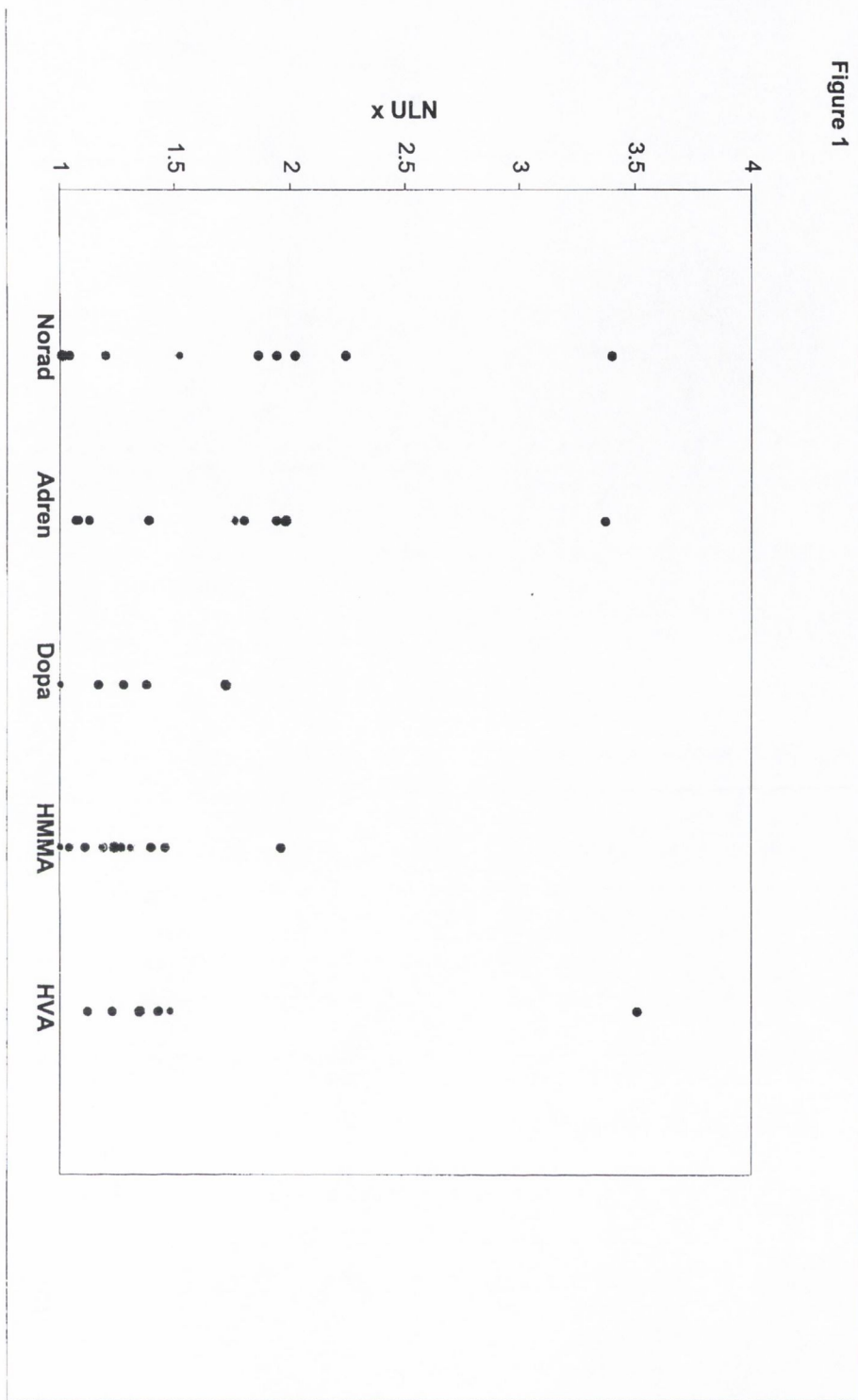


Figure 1 legend: False positive elevations in urine catecholamines, HMMA and HVA individual values from a total of 15 patients are shown as multiples of the upper limit of normal (ULN). For noradrenaline, there are 9 data points from 9 patients; for adrenaline, there are 9 data points from 8 patients; for dopamine, there are five points from 4 patients, for HMMA, 12 points from 8 patients and for HVA there are 7 points from 7 patients. The ULN for noradrenaline, adrenaline and dopamine are 900, 230 and 3300 nmol/24 hr respectively and for HMMA and HVA are 45 and 40 μ mol/24 hr respectively.

Discussion

Investigation for phaeochromocytoma is usually initiated by clinical suspicion but may be the result of screening of family members in an indicative case¹⁵. In the past diagnosis was made at autopsy in 40% to 76%^{16,17}. Histological confirmation is the ultimate diagnostic gold standard. Currently, biochemical screening of urinary catecholamines, or of metanephrine and normetanephrine, is considered the first option, followed by imaging. The most efficient imaging modality for the localisation of intra-adrenal sites in phaeochromocytoma appears to be CT and MRI. MRI is complementary to MIBG in the abdomen and pelvis. However, there is considerable overlap between the MR appearance of phaeochromocytoma and other adrenal lesions and a recent study found that MR imaging had a sensitivity of 64.7% and a specificity of 88% in the diagnosis of adrenal phaeochromocytomas¹⁸. Continuous rotation and/or spiral CT give better spatial resolution than MRI in the thorax^{19,20}. Because it is necessary to search for extra-adrenal tumour sites, it is suggested that MIBG be performed first because it offers whole body coverage. MIBG is superior for the localisation of suspected recurrence after surgery and is also useful for the detection of extra-adrenal tumours²¹. The isotope label used with MIBG is important. The superior counting statistics of the 159 keV photon of ¹²³I MIBG in comparison to the 364 KeV photon of ¹³¹I have permitted visualisation of phaeochromocytomas not seen with ¹³¹I MIBG. However, the short half life of ¹²³I, reduces its shelf life so much that it must be synthesized on the day of injection and is therefore more costly²². Each imaging modality has its place and their association offers the best results. ¹¹¹In octreotide scans may also be complementary to MIBG in the localisation of phaeochromocytoma but its place is still under investigation²³. Clinical practice in the investigation of phaeochromocytoma is often imperfect and some tumours may be inadequately excluded². Certainty in tumour exclusion in some cases may be impossible where the urine biochemistry remains elevated yet MIBG, CT and MRI scans are negative. However as about 90% of tumours are intra-adrenal, CT alone is likely to be adequate in most. As an untreated phaeochromocytoma may cause death from an intracranial bleed, cardiac infarction, arrhythmia or arrest and shock, discovery is important. The disease is curable with surgical removal where the operative mortality is from 0 to <3%²⁴. The use of MIBG in the investigation of phaeochromocytoma has been increasing quickly in this hospital. Abdominal ultrasound with a published sensitivity of 89% is also a cheap, convenient and commonly used technique²⁵. Unless other pathology is suspected, IVP and renography is unnecessary. Despite published protocols, systematic investigation is difficult to ensure in routine clinical practice. It is clear that physicians here place much reliance on the MIBG scan as a disease excluder despite its limitations. Using a subsequent positive diagnosis as the standard, no false negative examinations have been seen to date. Some centres use MIBG only when bilateral or extra-adrenal phaeochromocytomas are suspected²⁶ whereas others use ultrasound, CT, MRI and MIBG to localise a tumour following laboratory diagnosis²⁵. Raised biochemical marker values invariably result in imaging investigations but one quarter of the MIBG scans in this series were performed for clinical reasons despite a negative biochemical screen. The use of biochemical reference values derived from non-phaeochromocytoma hypertensive patients which are higher than those from normotensives helps to limit the numbers of false positives and therefore the costs of investigations. The provision of information on current drug therapy on each patient is important to prevent reporting of false positives due to analytical or pharmacological interferences²⁷. Therapeutic drugs such as tricyclic antidepressants, neuroleptics, labetalol and sympathomimetics may affect the neuronal MIBG uptake. These should be considered when interpreting negative MIBG scans. Phenoxybenzamine, propranolol, prazosin and calcium antagonists do not interfere with the MIBG uptake.

Metanephrines had been used as primary analytes in some institutions whereas others found their diagnostic usefulness similar to free catecholamines^{28, 29}. In this institution, the published sensitivity and specificity for catecholamines and HMMA together in the diagnosis of pheochromocytoma is 82% and 93.2% respectively². Enzyme immunoassays (ELISA) for urine metanephrine and normetanephrine have recently been developed³⁰. Analyser technology for ELISA assays is widely available and easy to use. This should make screening simpler, less time consuming and cheaper. Ultimately, the investigation protocol in each hospital will depend upon the availability and cost of the local laboratory and imaging services.

Over the period 1990 – 1997, during which the annual number of biochemical screens for pheochromocytoma rose from 342 in 1990 to 837 in 1997, there were another 16 cases of pheochromocytoma diagnosed without using scintiscanning. The role of MIBG scanning in searching for second tumours and metastases seems under recognised despite the published evidence.

References

1. Stein PP, Black HR. A simplified approach to pheochromocytoma. A review of the literature and report on one institution's experience. *Medicine* 1990;70:46-66.
2. Tormey WP, FitzGerald RJ. Lack of uniformity in the clinical approach to the interpretation of urinary catecholamines and their metabolites. *Ir J Med Sci* 1995;164:146-50.
3. Cheung PSY, Thompson NW, Dmuchowski CF, Sisson JC. Spectrum of pheochromocytoma in the ¹³¹I-MIBG era. *World J Surg* 1988;12:546-51.
4. Chatal JF, Charbonnel B. Comparison of iodobenzylguanidine imaging with computed tomography in locating pheochromocytoma. *J Clin Endocrinol Metab* 1985;61:769-72.
5. Maurea S, Cuocolo A, Reynolds JC, Tumeh S, Begley MG, Linehan WM, Norton JA, McClellan MW, Keiser HR, NeuMann RD. Iodine-131-metaiodobenzylguanidine scintigraphy in preoperative and postoperative evaluation of paragangliomas; comparison with CT and MRI. *J Nucl Med* 1993;34:173-9.
6. Shapiro B, Copp JE, Sisson JC, Eyre PL, Wallis J, Beierwaites WH. Iodine-131 metaiodobenzylguanidine for the locating of suspected pheochromocytoma: experience in 400 cases. *J Nucl Med* 1985;26:576-85.
7. Plouin P-F, Degoulet P, Tugaye A, Ducrocq M-B, Menard J. Le depistage du pheochromocytome: chez quels hypertendus? Etude semiologique chez 2585 hypertendus dont 11 ayant un pheochromocytome. *Nouv Presse Med* 1981;10:869-72.
8. Sclafani LM, Woodruff JM, Brennan MF. Extraadrenal retroperitoneal paragangliomas: Natural history and response to treatment. *Surgery* 1990;108:1124-30.

9. Melicow MM. One hundred cases of pheochromocytoma (107 tumors) at the Columbia presbyterian Medical Center, 1926 – 1976. *Cancer* 1977;40:1987-2004.
10. O'Neill G, Tormey WP, Farrell MA, FitzGerald RJ, Finucane J. Phaeochromocytoma and lethal lactic acidemia. *Ir Med J* 1993;86:221.
11. Wieland DM, Wu J, Brown LE, Mangner TJ, Swanson DP, Beierwaltes WH. Radiolabeled adrenergic neuron-blocking agents: adrenomedullary imaging with [¹³¹I]iodobenzylguanidine. *J Nucl Med* 1980;21:349-53.
12. Sisson JC, Wieland DM, Sherman P, Mangner TJ, Tobes MC, Jacques S. Metaiodobenzylguanidine as an index of the adrenergic nervous system integrity and function. *J Nucl Med* 1987;28:1620-4.
13. FitzGibbon M, FitzGerald RJ, Tormey WP, O'Meara A, Kenny D. Reference values for urinary HMMA, HVA, noradrenaline, adrenaline and dopamine excretion in children using random urine samples and HPLC with electrochemical detection. *Ann Clin Biochem* 1992;29:400-4.
14. Davidson DF, FitzPatrick J. A simple, optimised and rapid assay for urinary free catecholamines by HPLC with electrochemical detection. *Ann Clin Biochem* 1985;22:297-303.
15. Neumann HPH, Berger DP, Sigmund G, Blum U, Schmidt D, Parmer RJ, Volk B, Kirste G. Pheochromocytomas, multiple endocrine neoplasia type 2, and Von Hippel-Lindau disease. *N Engl J Med* 1993;329:1531-8.
16. St John Sutton MG, Sheps SG, Lie JT. Prevalence of clinically unsuspected pheochromocytoma. Review of a 50-year autopsy series. *Mayo Clin Proc* 1981;56:354-60.
17. Stenstrom G, Svardsudd K. Pheochromocytoma in Sweden 1958-1981. *Acta Med Scand* 1986;220:225-32.
18. Varghese JC, Hahn PF, Papanicolaou N, Mayo-Smith WW, Gaa JA, Lee MJ. MR differentiation of phaeochromocytoma from other adrenal lesions based on qualitative analysis of T2 relaxation times. *Clin Radiol* 1997;52:603-6.
19. Davis SD. CT evaluation for pulmonary metastases in patients with extrathoracic malignancy. *Radiology* 1991;180:1-12.
20. Webb WR, Sostman HD. MR imaging of thoracic disease: clinical uses. *Radiology* 1992;182:622-30.
21. Chatal J-F. Can we agree on the best imaging procedure(s) for localization of pheochromocytomas ? *J Nucl Med* 1993;180:1.

22. Lynn MD, Shapiro B, Sisson JC, Beierwaltes WH, Meyers LJ, Ackerman R, Mangner TJ. Pheochromocytoma and the normal adrenal medulla: improved visualization with I-123 MIBG scintigraphy. *Radiology* 1985;155:789-92.
23. Kopf D, Bockisch A, Steinert H, Hahn K, Beyer J, Neumann HP, Hensen J, Lehnert H. Octreotide scintigraphy and catecholamine response to an octreotide challenge in malignant phaeochromocytoma. *Clin Endocrinol* 1997;46:39-44.
24. Juan D. Pharmacologic agents in the management of pheochromocytoma. *South Med J* 1982;75:211-6.
25. Lucon AM, Pereira MAA, Mendonca BB, Halpern A, Wajchenbeg BL, Arap S. Pheochromocytoma: study of 50 cases. *J Urol* 1997;157:1208-12.
26. Loh KC, Shlossberg AH, Abbott EC, Salisbury SR, Tan MH. Phaeochromocytoma: a ten year survey. *Q J Med* 1997;90:51-60.
27. Bravo EL. Evolving concepts in the pathophysiology, diagnosis and treatment of pheochromocytoma. *Endocrine Reviews* 1994;15:356-68.
28. Heron E, Chatellier G, Billaud E, Foos E, Plouin P-F. The urinary metanephrine-to-creatinine ratio for the diagnosis of pheochromocytoma. *Ann Intern Med* 1996;125:300-3.
29. Graham PE, Smythe GA, Edwards GA, Lazarus L. Laboratory diagnosis of phaeochromocytoma: Which analytes should we measure? *Ann Clin Biochem* 1993;30:129-34.
30. Wolthers BG, Kema IP, Volmer M, Wesemann R, Westermann J, Manz B. Evaluation of urinary metanephrine and normetanephrine enzyme immunoassay (ELISA) kits by comparison with isotope dilution mass spectrometry. *Clin Chem* 1997;43:114-20.

The excretion of catecholamines, HMMA and HVA in patients with elevated urinary 5-hydroxyindoleacetic acid and their clinical associations.

Carcinoid tumours

Carcinoid tumours derive from enterochromaffin cells which are found in the gastrointestinal system including the salivary glands, pancreas and biliary tree, and in the bronchial tree, thyroid, ovary, thymus or uterus. Carcinoids have a reported frequency of about 3.2 to 15 cases per million population per year¹⁻³. They may be found incidentally in 0.50% to 0.75% of autopsies⁴. Despite an expected annual incidence of 20 new cases per year, a 15 year study in Northern Ireland reported an annual incidence of 13 per million of the population for carcinoid tumour and no patient presented with the carcinoid syndrome¹. They are slow growing and the average time lapse between symptoms and diagnosis is 4.5 years. 5-hydroxytryptamine (5-HT) production by these tumours depends on the site of origin. Ileal and other mid-gut carcinoids secrete large quantities of 5-HT which are then metabolised to 5-hydroxyindoleacetic acid (5-HIAA) and excreted in the urine. Fore-gut tumours such as bronchial, pancreatic, duodenal or biliary carcinoids secrete mainly 5-hydroxytryptophan (5-HTP) which is metabolised to 5-HT and 5-HIAA at other sites. In carcinoids that present with carcinoid syndrome, liver metastases are present in about 95% except where the primary site is in the lungs or ovaries. Carcinoids may produce histamine, bradykinin, prostaglandins, enkephalins and endorphins, gastrin, motilin, neurotensin, arginine vasopressin, chorionic gonadotropin, insulin, adrenocorticotrophic hormone and catecholamines².

Flushing and diarrhoea are the usual symptoms associated with carcinoid tumour that precipitate a laboratory estimation of 5-HIAA. Up to 5% of new onset flushing may be caused by the carcinoid syndrome and about 5% of patients with a carcinoid tumour develop symptoms of carcinoid syndrome. This depends on the site of the tumour with 30 to 60% of small bowel tumours, 1% of appendix and almost no rectal carcinoids causing symptoms. With gut carcinoids, the syndrome only develops when liver metastases occur and in one series 90% of tumours were not suspected *ante mortem*⁵. Ninety per cent of patients with carcinoid syndrome complain of flushing. Flushing is not 5-HT mediated and substance P, neuropeptide K, neurokinin A, bradykinin, prostaglandins and histamine are among the candidate mediators^{2,6,7}. Gastric carcinoids account for 3% of gut carcinoids and the flush from these rare tumours is histamine mediated⁸. A drug history is important to avoid unnecessary laboratory testing. Diarrhoea occurs in 75% of carcinoid syndromes and 5-HT inhibition reduces the episodes. Bronchospasm is also a feature in about 20%. There is an association of neuro-fibromatosis, phaeochromocytoma and somatostatin-rich duodenal carcinoid syndrome⁹. The spectrum of clinical symptoms in carcinoid is wide.

Carcinoids and phaeochromocytomas have their origins in the neural crest. Carcinoid tumours containing tryptophan hydroxylase secrete 5-HT and phaeochromocytomas

containing tyrosine hydroxylase may secrete dopamine, noradrenaline and adrenaline. Feldman reported that an increased urinary HVA and dopamine excretion occurred in 18% and 27% respectively of 45 carcinoid tumours that produced 5-HT¹⁰. During acute episodes of flushing associated with carcinoids, blood pressure falls 20 to 30 mmHg and noradrenaline, adrenaline and dopamine are thought to provoke attacks of flushing and hypotension in patients with carcinoid tumours^x.

Study

The clinical and laboratory associations of the 24 cases from 5.5 years of laboratory work where at least one 24 hour urinary 5-HIAA was elevated are examined.

Methods

The 298 laboratory requests for urinary 5-HIAA over a five and one half year period from 1989 were searched to identify raised values. These occurred in 43 samples from 24 patients. These were 13 women and 11 men, ranging in age from 15 to 84 years with a median age of 57 years. The principal symptoms which triggered the laboratory requests were determined by perusal of the case notes. The ward dispensing records were examined to establish what drugs were dispensed to the patients during the period of the urine collection. The urine collections were considered complete when the volume was greater than 500 ml. It proved impossible to establish retrospectively whether the patients had consumed bananas, pineapples or other fruits which may cause minor elevations in urinary excretion of 5-HIAA.

5-HIAA was measured by HPLC with electrochemical detection as described in the methods section in Chapter 4. The between batch coefficient of variation (n=20) was 15.3% at 25 $\mu\text{mol/L}$ and 6.4% at 155 $\mu\text{mol/L}$. Drug interferences may arise. The application of a detector voltage of +0.67 volts was used as neither atenolol nor paracetamol interfere with the assay at this detector voltage. The reference range is 8 – 47 $\mu\text{mol/24 hours}$.

Results

HMMA values corresponding to a raised 5-HIAA level were available from the 35 collections where they had been requested and five (14.3%) of these HMMA values were greater than the upper reference limit of 45 $\mu\text{mol/24 hours}$. Levels of homovanillic acid (HVA) corresponding to an increased 5-HIAA were available from 28 collections and six (21%) of these were above the upper reference limit of 40 $\mu\text{mol/24 hours}$. Catecholamines were measured in 12 of the 24 patients with raised 5-HIAA values and normal values were found in six (50%). Two of the raised values were from patients who proved to have pheochromocytoma. The clinical diagnoses are shown in the Table 1. The four other patients with elevated urinary catecholamines were numbers 10, 12, 13 and 20 as listed in the table. Two of these complained of flushing. Case 10 was a patient from a psychiatric hospital with malignant neuroleptic syndrome where HMMA, HVA and all three catecholamines

were elevated but where the catecholamines returned to normal and the metabolites to near normal within days. No diagnostic imaging was performed which makes the diagnosis uncertain. Case 12 was a female patient with a large renal cell carcinoma who had hypertension and was taking nifedipine, doxazosin, glibenclamide and cimetidine. Calcium channel blockers can lead to a reflex increase in catecholamine release and may also cause flushing. Case 13 was taking atenolol and abdominal CT and ultrasound scans were normal. Case 20 was a 54-year old with cardiac failure from valvular heart disease who had episodes of hypertension, flushing and sweating and an altered bowel habit. The noradrenaline excretion was raised at 1292 $\mu\text{mol}/24$ hr. No abdominal imaging was performed and therefore phaeochromocytoma was not formally excluded.

Table The clinical correlations of the raised 5-HIAA values. Pre- and post-op indicate the timing of the urine collection relative to surgery

<i>Case and diagnosis</i>	<i>5-HIAA level ($\mu\text{mol}/24\text{ h}$)</i>
1 carcinoid tumour	608 (pre-op) 79 (post-op) 78 (post-op) 63 (post-op)
2 carcinoid tumour	822 (post-op)
3 carcinoid tumour	121 (post- chemotherapy)
4 carcinoid tumour	234 324
5 phaeochromocytoma	85 (post-op)
6 phaeochromocytoma	73 (pre-op)
7 gut hormone secreting tumour	58
8 possible adrenal mass on ultrasound	81.6
9 neurofibromatosis with a possible carcinoid tumour	53 45.4
10 malignant neuroleptic syndrome	176 69 19
11 Klippel Trenaunay syndrome	51
12 hypernephroma – poor anaesthetic risk	95 79
13 diabetes mellitus with flushing and hypertension	84 136 14 31
14 flushing associated with a calcium channel blocker	60
15 flushing associated with a calcium channel blocker	55.4
16 asthma and theophylline	56.7
17 possible carcinoid tumour	83.2 89.6 84.0
18 palpitations associated with a patent foramen ovale	59.4 20.3 11.1 11.6 20.7
19 palpitations and flushing	72.2
20 flushing, sweating with cardiac failure from valvular heart disease	66.1
21 flushing	96.2
22 flushing	82.6
23 episodic diarrhoea with pituitary disease	85.5
24 hypertension	56

A brief summary of the other patients listed in table 1 follows. Three of the four carcinoid tumours were histologically confirmed. The primary site was ileum in two, ileum in one and unknown in the other. Each had liver metastases. In Case 1, there was a raised HMMA of 66 $\mu\text{mol/L}$ and HVA of 48 $\mu\text{mol/L}$. These were normal in the other three.

In case 7, the patient had watery diarrhoea, a facial rash with flushing and periodic hypotension. Gut hormones were negative and glucagonoma was specifically excluded. CT and ultrasound were negative and a previous 5-HIAA had been normal. In Case 8, weight loss, diarrhoea, episodic asthma, oesophagitis and hypertension were present but CT, MIBG scans and urinary catecholamines were negative. Lisinopril and ranitidine was the drug therapy and the cause of the 5-HIAA of 81.6 remained unknown. In case 9, sweating flushing and occasional palpitations were present. Telangiectasia and an acoustic neuroma were present. Diltiazem was used but carcinoid tumour remains a possibility. In case 11, a 19-year old with flushing, sweating, vague abdominal discomfort, telangiectasia and lower limb varicosities were noted. An abdominal, ultrasound was performed and found to be normal. In cases 14 and 15, nifedipine and verapamil therapy for hypertension were the likely causes and theophylline in asthma treatment may have been a factor in case 16. In case 17, a CT scan and urinary free catecholamines were negative but flushing, palpitations and rash were persistent symptoms and carcinoid remains a possibility. Case 18 had a normal CT scan and the initial value was coincidental. Case 19 was a 39 year old with flushing, palpitations from atrial fibrillation and obesity. Digoxin was prescribed and carcinoid considered unlikely. Paroxysmal flushes and pyelonephritis were the presenting problems in case 21 but a second specimen was normal. In case 22, flushes and abdominal pain in a 15 year old with ureteric implants and renal disease were diagnosed following imaging. Case 23 was a 23 year old with episodic diarrhoea, reflux oesophagitis and weight loss who had diabetes insipidus and was receiving growth hormone replacement therapy for pituitary failure. Case 24 was a hypertensive marathon runner taking captopril and thiazides. Firm conclusions cannot be drawn in many of these cases as to whether the increased 5-HIAA is a coincidence or an indicator of disease.

Associated with the raised urinary 5-HIAA levels were increased levels of HMMA in 14.3% of the 35 collections where it was measured and increased levels of HVA in 21% 28 collections where measured.

The drugs dispensed during the period of the urine collections are listed in table 2 below.

Table 2.

H₂-blockers (cimetidine, ranitidine)
 Antihypertensives (bendrofluazide, bumetanide*, 'frumil' frusemide and amiloride*)
 Alpha blocker (doxazosin)
 Beta-blocker (atenolol)

Calcium channel blockers (verapamil, nifedipine*, diltiazem)
ACE inhibitors (captopril, lisinopril,)
Digoxin*
Anti-arrhythmic (propafenone)
Nitrate (isosorbide mononitrate)
Platelet adhesiveness inhibitors (aspirin, dipyridamole)
Nonsteroidal anti-inflammatory (diclofenac, naproxen)
Morphine sulphate
glibenclamide
finasteride
chlorpromazine
benzodiazepines (temazepam, fluorazepam)
Bronchodilators (theophylline) inhalers (oxitropium bromide, salbutamol, beclomethasone)

* dispensed twice

Discussion

Flushing and diarrhoea are the usual symptoms associated with carcinoid tumour which precipitate a laboratory request for urinary 5-HIAA. About 75% of patients with carcinoid syndrome excrete $>80 \mu\text{mol}$ of 5-HIAA per day and a diagnostic specificity of 100% is claimed for this level of excretion provided that the dietary level of fruits and drugs is controlled². False positive increases in 5-HIAA may result from the consumption of bananas, pineapple, kiwi fruit, plums, pecans, avocados and also paracetamol. Aspirin and L-dopa may depress 5-HIAA levels. A diagnostic specificity of 100% is claimed for the above level if diet is controlled². The findings here do not confirm this assertion.

Benzodiazepines, naproxen, atenolol, chlorpromazine and possibly other drugs were all potential interfering substances in these patients and drug-free specimens are the ideal. Urinary 5-HIAA may be lowered in renal failure. Analytical imprecision at low values near the reference value limit may have contributed to some of the slightly elevated values that were normal on repeat testing. Minor inadvertent prolongation of the urine collection period may also be a factor. The assay performance for higher values is well within the desired analytical goal of $<10\%$ as a between batch coefficient of variation¹¹. Negative 5-HIAA results when carcinoid is strongly suspected may occur if 5-hydroxytryptophan and 5-hydroxytryptamine are not being metabolised, or if the 5-HIAA secretion is intermittent¹¹.

Our figures indicate that raised urinary HMMA and HVA values are not rare when 5-HIAA excretion is increased irrespective of the apparent underlying cause. There is no agreed threshold value of urinary 5-HIAA excretion that triggers the performance of a radionuclide scan, even though such imagery will detect up to 86% of carcinoid tumours. As well as CT and barium contrast studies, radiolabelled MIBG, vasoactive intestinal peptide and octreotide scans are used to try to localise carcinoids.

Addenda – In 1999, a 39 year old patient presented with an greatly enlarged liver and was found to have a metastatic carcinoid tumour. His liver biochemistry showed a plasma bilirubin 41 $\mu\text{mol/L}$, ALT 637 iu/L , alkaline phosphatase 771 iu/L , all elevated. The 5-HIAA was 1877 while the HMMA 25.9 and HVA 37.6. A tumour biopsy was positive for chromogranin, Cam 5.2 and AE1 AE3. Despite the large tumour mass, the catecholamine metabolites remained normal.

In the total list of phaeochromocytoma patients to November 1999, 5-HIAA was measured pre-operatively in 7 patients with phaeochromocytoma. Two of these (28%) had raised values of 85 and 72.9 $\mu\text{mol/24 hours}$ respectively. Neither had flushing as a symptom.

References

1. Watson RGP, Johnston CF, O'Hare MMT, Anderson JR, Wilson BG, Collins JSA, Sloan JM, Buchanan KD. The frequency of gastrointestinal endocrine tumours in a well-defined population – Northern Ireland 1970-1985. *Q J Med* 1989;72:647-57.
2. Kaplan LM. Endocrine tumors of the gastrointestinal tract and pancreas. In: Isselbacher KJ, Braunwald E, Wilson JD, Martin JB, Fauci AS, Kasper DL, eds. *Harrison's Principles of Internal Medicine*. 13 edn New York: McGraw Hill, 1994;pp1535-42.
3. Anon Octreotide *Lancet* 1989;ii:541-2.
4. Lightdale CJ. Tumors of the small and large intestines. In: Stein JH ed. *Internal Medicine*, 4th edn. St Louis: Mosby, 1994;pp475-83.
5. Berge T, Linell F. Carcinoid tumors: frequency in a defined population during a 12 year period. *Acta Pathol Microbiol Scand* 1976;84:322-30.
6. Norheim I, Theodorsson-Norheim E, Broden E, Oberg K. Tachykinins in carcinoid tumors: their use as a tumor marker and possible role in the carcinoid flush. *J Clin Endocrinol Metab* 1986;63:605-12.
7. Oates J. The carcinoid syndrome. *N Engl J Med* 1986;315:702-4.
8. Roberts LJ II, Marney SR Jr, Oates JA. Blockade of the flush associated with metastatic gastric carcinoid by combined histamine H_1 and H_2 receptor antagonists: evidence for an important role of H_2 receptors in human vasculature. *N Engl J Med* 1979;300:236-8.
9. Griffiths DFR, Williams GT, Williams ED. Multiple endocrine neoplasia associated with von Recklinghausen's disease. *BMJ* 1983;287:1341-3.

10. Feldman JM. Increased dopamine production in patients with carcinoid tumors. *Metabolism* 1985;34:255-260.
11. Deacon AC. The measurement of 5-hydroxyindoleacetic in urine. *Ann Clin Biochem* 1994;31:215-32.
12. Whitley RJ, Meikle AW, Watts NB. Endocrinology Part IV, serotonin and 5-hydroxyindoleacetic acid. In: Burtis CA, Ashwood ER, eds. *Tietz textbook of clinical chemistry*. Philadelphia: WB Saunders, 1994;pp1765-75.

Renal cell carcinoma presenting as a pseudo-carcinoid tumour

Summary

A 38 year old woman presented with hypertension and a renal cell carcinoma. Raised urinary 5-HIAA values $>300 \mu\text{mol/L}$, predictive of carcinoid tumour were found during the perioperative period. The values returned towards normal two weeks post surgery. Immunohistochemical examination of the renal cell carcinoma with neuroendocrine markers was negative. Despite a diligent search, no carcinoid tumour could be identified and the patient was well four years later. There was no dietary or drug explanation for this pseudocarcinoid.

Introduction

Renal cell carcinomas may occur sporadically or may be part of the autosomal dominant von Hippel-Lindau disease where phaeochromocytoma plays a part¹. The occurrence of other primary malignancies is common in patients with renal cell carcinomas. These may be antecedent, synchronous or subsequent to the diagnosis of the renal cell tumour². The interval between the onset of symptoms and diagnosis in carcinoid tumours averages about 4.5 years. Determination of urinary 5-hydroxyindoleacetic acid (5-HIAA) is the mainstay of the laboratory diagnosis of carcinoid tumours. The main sites of 5-HIAA synthesis are the liver and kidney. 5-HIAA is water soluble and is excreted almost entirely in the urine³. We report a patient with renal cell carcinoma who is alive and well four years after surgery despite having a raised urinary 5-HIAA values which indicated a strong probability of carcinoid tumour but where no such tumour could be found.

Case Report

A 38 year old female presented with left loin pain and a low grade pyrexia of 38°C associated with a sudden increase in size in a left hypochondrial swelling which had been present for several years. Blood pressure was 170-220mmHg systolic and 100-110mmHg diastolic, plasma creatinine was $83 \mu\text{mol/L}$ and Hb was 8.0g/dl. Abdominal ultra sound and CT scan confirmed a large left renal mass. An isotope bone scan was negative. Nifedipine was given to control the hypertension. A screen for phaeochromocytoma and carcinoid tumour, using HPLC with electrochemical detection⁴ revealed normal urine catecholamines and a slightly increased HVA with a greatly elevated 5-HIAA only. A second collection five days after surgery gave a similar result. However in later specimens collected more than two weeks post surgery, the 5-HIAA values were dramatically lower. Details of the urine biochemistry are shown in Table 1. Dietary causes of an increased excretion of 5-HIAA such as bananas, pineapples, kiwi fruits, plums, avocados and walnuts were excluded. However 'solpadeine' (paracetamol, codeine and caffeine) was administered postoperatively for pain and atenolol, captopril and allopurinol were the additional drugs taken between the first and latter pair of urine collections. These drugs do not interfere with the assay for 5-HIAA and thus, drug interference is a most unlikely cause. During the performance of a left radical nephrectomy, paroxysms of hypertension and tachycardia occurred during the operative manipulation of the renal mass which was confirmed histologically as a grade 2 renal parenchymal cell carcinoma. Immunohistochemical stains for neuro-endocrine markers including

neuron specific enolase, chromogranin, synaptophysin and neurofilament were negative.

Postoperatively, dependent oedema with a plasma albumin of 21g/L occurred. There was a short episode of diarrhoea from the 3rd to the 5th postoperative day. By the 12th postoperative day, a gastric outflow obstruction due to an intramural duodenal haematoma caused pain and vomiting but settled over 15 days following conservative treatment. Parenteral nutrition was instituted during this period. Recovery was complete and she was discharged on the 40th day post surgery. At a four year follow-up, the patient was asymptomatic with a negative bone scan and had returned to work.

Table 1

	Noradrenaline (50 - 900)	Adrenaline (25 - 230)	Dopamine (250 - 3300)	HMMA (< 45)	HVA (< 40)	5-HIAA (< 50)
Pre-Op	240	120	441	28.1	48.5	339.2
Post-Op						
Day 5						324
Day 15						60
Day 18	717	474	1065	28.2	25.6	71.7

Legend

Pre and post operative 24hr urine values. Reference ranges are bracketed. Units for noradrenaline, adrenaline and dopamine are nmol/24hr, and for HMMA, HVA and 5-HIAA are µmol/24hr.

Discussion

In about 75% of patients with carcinoid syndrome, 5-HIAA excretion is >80µmol/24hr and the specificity of such raised values for carcinoid is regarded as approximately 100% when 5-hydroxytryptamine containing foods are avoided⁵. The high urinary 5-HIAA values here are considerably greater than the maximum levels induced by dietary manipulation and the rapid reduction in values from a diet induced peak indicates that persistent systemic production is necessary for prolonged urinary excretion⁶. Patients with 5-HT producing tumours may have raised levels of dopamine or its metabolite HVA⁷ as in this patient. Acute catecholamine release from carcinoid tumours may be sufficient to raise blood pressure⁸ but hypertension has been reported in 37% of patients with renal cell carcinoma⁹. Thus, there were a number of possible contributory factors to this patient's hypertension. With raised blood pressure and a

duodenal intramural mass, duodenal carcinoid had to be considered as there is an association with pheochromocytoma and neurofibromatosis¹⁰.

Scintigraphy with radiolabelled octreotide or metaiodobenzylguanidine (MIBG) is used where a carcinoid tumour or a pheochromocytoma is difficult to detect. A preoperative MIBG scan was negative in this patient. Why were there such high urinary 5-HIAA values in a patient with renal cell carcinoma and why the values were still raised five days following surgery only to dramatically fall within a fortnight remains unresolved?

References

1. Neumann HP, Bender BU, Berger DP, Laubenberger J, Schultze-seemann W, Wetterauer U, Ferstl FJ, et al Prevalence, morphology and biology of renal cell carcinoma in von Hippel-Lindau disease compared to sporadic renal cell carcinoma. *J Urol* 1998;160:1248-54.
2. Rabani F, Grimaldi G, Russo P. multiple primary malignancies in renal cell carcinoma. *J Urol* 1998;160:1255-1259.
3. Deacon AC. The measurement of 5-hydroxyindoleacetic acid in urine. *Ann Clin Biochem* 1994;31:215-232.
4. Davidson DF. Simultaneous assay for urinary 4-hydroxy-3-methoxy-mandelic acid, 5-hydroxyindoleacetic acid and homovanillic acid by isocratic HPLC with electrochemical detection. *Ann Clin Biochem* 1989;26:137-143.
5. Kaplan LM. Endocrine tumors of the gastrointestinal tract and pancreas. In: Isselbacher KJ, Braunwald E, Wilson JD, Martin JB, Fauci AS, Kasper DL, eds. *Harrison's Principles of Internal Medicine*, 13th edn. New York:McGraw Hill, 1994:1535-42.
6. Kema IP, Schellings AMJ, Meiborg G, Hoppenbrouwers CJM, Muskiet FAJ. Influence of a serotonin- and dopamine-rich diet on platelet serotonin content and urinary excretion of biogenic amines and their metabolites. *Clin Chem* 1992;38:1730-6.
7. Feldman JM. Increased dopamine production in patients with carcinoid tumors. *Metabolism* 1985;34:255-60.
8. Kema IP, de Vries EGE, Slooff MJH, Biesma B, Muskiet FAJ. Serotonin, catecholamines, histamine, and their metabolites in urine, platelets, and tumor tissue of patients with carcinoid tumors. *Clin Chem* 1994;40:86-95.
9. Farrow GM. Diseases of the kidney. In: Murphy WM ed. *Urological Pathology*, Philadelphia:W.B.Saunders, 1989:409-82.
10. Wheeler MH, Curley IR, Williams ED. The association of neurofibromatosis, pheochromocytoma, and somatostatin-rich duodenal carcinoid tumor. *Surgery* 1986;100:1163-68.

Multiple Endocrine Neoplasia (MEN)

The cell type involved in these tumours is usually composed of one or more specific polypeptide and biogenic amine producing cell types called APUD (Amine Precursor Uptake and Decarboxylation). The APUD cell type is thought to derive embryologically from neural crest cells. The lipomas of MEN1 and the ganglioneuromas of MEN Type 2B are the exception. These tumours may progress from hyperplasia to adenoma and in some cases to carcinoma. The tumours are multicentric in origin and are autosomal dominant¹.

Families with familial pheochromocytoma may have evidence of MEN1, MEN 2A, MEN 2B, von Hippel-Lindau retinocerebellar haemangioblastosis or von Recklinghausen's neurofibromatosis.

The pheochromocytomas in MEN 2A usually arise from adrenal medullary hyperplasia and are usually multicentric and often bilateral. Extra-adrenal pheochromocytomas are unusual in this situation.

MEN Type 1

Hyperparathyroidism

Pancreatic Islet Cell tumours – Gastrinomas, insulinomas, glucagonomas, watery diarrhoea syndrome (Werner-Morrison), pancreatic polypeptide.

Pituitary adenomas – Prolactinoma, GH/GHRH tumours, Cushing's syndrome and CRH tumours

Carcinoid tumours (uncommon feature)

Other minor associations

Subcutaneous and visceral lipomas occur in some patients and cutaneous leiomyomas may occur.

Genetics – The causative tumour suppressor gene "menin" has been localised to chromosome 11q13².

MEN Type 2

- Type 2A – **Medullary thyroid carcinoma**
- **Phaeochromocytoma** (>50% develop unilateral or bilateral Phaeos)
 - **Hyperparathyroidism** (10% to 35%)
 - **2A Variants**
 - Familial medullary thyroid carcinoma
 - 2A with cutaneous lichen amyloidosis variant
 - 2A with Hirschsprung's disease
 - 2A with ovarian strumal carcinoid
- Type 2B – **Medullary thyroid carcinoma**
- **phaeochromocytoma** in 50%
 - **hyperparathyroidism** (rare)
 - **multiple mucosal neuromas and ganglioneuromas**
 - **marfanoid habitus, pectus excavatum, slipped femoral epiphysis.**

About 50% of MEN 2B patients exhibit the complete syndrome with mucosal neuromas in all affected patients. These are most common in the lips, tongue and buccal mucosa.

Genetics – Gene for MEN 2 is on centromeric region of chromosome 10q11.2. This region contains the c-RET proto-oncogene which codes for a tyrosine kinase receptor. Mulligan et al and Donis-Keller et al independently identified point mutations of the RET proto-oncogene in MEN 2A and Familial Medullary Thyroid Cancer^{3,4}. Point mutations in the RET proto-oncogene have been shown in MEN 2B^{5,6}, MEN 2A/Hirschsprung's disease variant^{7,8}, MEN 2A/cutaneous lichen amyloidosis⁹ and Hirschsprung's disease^{10,11}. There have been no examples of a person with MEN 2 or FMTC in a family with a RET proto-oncogene mutation who does not have the mutation.

Genetics and phaeochromocytoma - Phaeochromocytoma and parathyroid adenomas are most commonly found with a codon 634 mutation of the RET proto-oncogene¹. Phaeochromocytomas and parathyroid neoplasia has been found in 5 to 20% of kindred with codon 611, 618 and 620 mutations of RET¹²⁻¹⁴. Phaeochromocytoma has not been found in those with codon 609 mutation¹². Sporadic phaeochromocytomas have an 8% prevalence of these RET proto-oncogene mutations¹⁵⁻¹⁷.

RET proto-oncogene codes for a cell surface receptor tyrosine kinase that that is involved in the regulation of cell growth and differentiation. It is on chromosome 10q11.2. The mutation for MEN 2A affects the coding for cysteine residues in the extracellular ligand-binding domain of the protein¹³. In MEN 2B, the mutations affect the tyrosine kinase catalytic site in the intracellular domain of the protein^{5,6,18}. A single point mutation in exon 16 of the RET proto-oncogene has been shown to cause MEN 2B in about 93% of patients^{5,6,12,18}. Sporadic phaeochromocytomas have a 6% prevalence of the MEN 2B type mutations¹⁵⁻¹⁷.

MEN 2A/phaeochromocytoma/biochemistry - Pheochromocytomas which form part of MEN 2A have been reported to have a distinctive biochemical pattern in which an increase in urine adrenaline and an increase in the ratio of urine adrenaline to noradrenaline are the first abnormalities noted¹⁹⁻²¹. Urinary HMMA is usually normal early in the course of the disease and has been reported to be unsuitable for prospective screening²². Less than 50% of pheochromocytomas in MEN 2A have sustained arterial hypertension. Later in the disease as the tumour enlarges, urinary noradrenaline, adrenaline, normetadrenaline and metadrenaline are elevated. Plasma catecholamine levels may be intermittently elevated early in the disease and are more sensitive but less specific than urine collections²³.

MEN of mixed type - Overlap syndromes

Carcinoid with MEN 1 or MEN 2

Pituitary tumours and pheochromocytomas +/- hyperparathyroidism

Gastrinomas in an MEN 2 patient

Adenomatous polyposis coli in Type 2B

Posterior pituitary tumour and MEN 1

Prolactinoma in MEN 2A

Pheochromocytoma in MEN 1

Von Hippel-Lindau disease (VHL) and pheochromocytoma

VHL is an autosomal dominant disease with haemangioblastomas of the CNS, retinal angiomas, renal cell carcinomas, visceral cysts (especially in the kidney and pancreas), pheochromocytomas, islet cell tumours, epidermal cystadenoma and endolymphatic sac tumours. Prior to surveillance protocols, the life expectancy of VHL patients was less than 50 years.

>90% of gene carriers have one or more of the above by age 60 years and more than 70% have a CNS tumour.

25% to 35% have unilateral or bilateral pheochromocytomas.

15% to 20% have islet cell tumours.

70% get renal cell cancers.

Variations in the disease cluster within families. Pheochromocytomas may occur in up to 90% in some families but may be rare in others²⁴. In some series, among patients with pheochromocytoma, up to 20% may have von Hippel-Lindau and the tumour may be multifocal and appear at an early age²⁵.

The VHL gene has been mapped to chromosome 3p25.3 and is a tumour suppressor gene. The disorder is caused by mutations or deletions in the gene. Mutation of codon 238 has been found in 40% of VHL families with pheochromocytomas and any patient with this mutation should be screened at intervals for pheochromocytoma²⁶. In type 2 VHL 5% to

20% of families have phaeochromocytomas. Most patients with phaeochromocytomas and VHL have missense mutations and one mutation carries a 62% risk of tumour occurrence²⁷. In VHL disease, 20% have large germline mutations detectable by Southern blot analysis, 27% have missense mutations, 27% have nonsense or frame shift mutations and in 20% no deletion or mutation can be found²⁸.

Some patients with sporadic phaeochromocytomas may have abnormalities on chromosome 3 near the VHL gene²⁹.

Phaeochromocytoma and islet cell tumours

This association may occur in familial^{30,31} or nonfamilial³²⁻³⁴ patterns and is very rare. The molecular genetics have not been elucidated.

Phaeochromocytoma and neurofibromatosis

Type 1 neurofibromatosis has neuromas along peripheral nerves whereas in Type 2, the neuromas are along the cochlear branch of the XIII cranial nerve.

Only neurofibromatosis type 1 has been associated with phaeochromocytoma, hyperparathyroidism, somatostatin-producing carcinoids of the duodenum, medullary thyroid carcinoma and hypothalamic tumours causing precocious puberty. The NF-1 gene is a putative tumour suppressor gene and is located on chromosome 17. The NF-1 gene encodes a RAS GTPase activating protein of 2818 amino acids called neurofibromin. It accelerates GTP hydrolysis on p21 RAS. Loss of GTPase-activating function of neurofibromin due to allelic loss or mutation leads to p21 RAS activation. In the mouse, this leads to sympathetic ganglion hyperplasia. There is evidence of loss of activity of this protein in NF-1 associated³⁵ and sporadic phaeochromocytomas³⁶. Defective or absent neurofibromin also induces constitutive activity in adenylate cyclase. Neurofibromatosis is autosomal dominant.

Reduced expression of neurofibromin may also occur in MEN 2A, MEN 2B and in VHL. These tumours show a predominant expression (60 – 75%) of a shorter form of neurofibromin resulting from alternative splicing. This isoform lacks 63 nucleotides corresponding to exon 23a of the NF-1 gene. The shorter form of neurofibromin may be related to a more intensely proliferative state of adrenal tissue, thereby contributing to tumour development with p21RAS activation³⁷.

Phaeochromocytoma is present in about 1% of type 1 patients but in neurofibromatosis patients with hypertension, it is about 50% or higher³⁸.

MEN 2 tumours may also be associated with the loss of tumour heterozygosity of chromosome 1p loci, which suggests the possible involvement of a recessive oncogene³⁸.

References

1. Gagel RF Multiple Endocrine Neoplasia In: Williams textbook of endocrinology 9th ed, Wilson JD, Foster DW, Kronenberg HM, Larsen Preds, WB Saunders Philadelphia. 1998:1627-49.
2. Chandrasekharappa SC, Guni SC, Manickam P, Olufami SE, Collins FS, Emmert-Buck MR, Debelenko LV, Zhuang Z et al. Positional cloning of the gene for multiple endocrine neoplasia type 1. *Science* 1997;276:404-7.
3. Mulligan LM, Kwok JBJ, Healey CS, Elsdon MJ, Eng C, Gardner E, Love DR, Mole SE, Moore JK, Papi L et al. Germline mutations of the RET proto-oncogene in multiple endocrine neoplasia type 2A (MEN2A). *Nature* 1993;363:458-60.
4. Donis-Keller H, Shenshen D, Chi D et al. Mutations in the RET proto-oncogene are associated with MEN 2A and FMTC. *Hum Mol Genet* 1993;2:851-6.
5. Carlson KM, Dou S, Chi D, et al. Single missense mutation in the tyrosine kinase catalytic domain of the RET protoncogene is associated with multiple endocrine neoplasia type 2B. *Proc Natl Acad Sci USA* 1994;91:1579-83.
6. Hofstra RM, Landsvater RM, Ceccherini I et al A mutation in the RET proto-oncogene associated with multiple endocrine neoplasia type 2B and sporadic medullary thyroid carcinoma. *Nature* 1994;367:375-6.
7. Lacroix A, Blanchard L, Villeneuve L et al Cosegregation of Hirschsprung's disease (HSCR) with chromosome 10 markers and a RET mutation in a French Canadian family with MEN 2A. *Abstr Fifth International Workshop on Multiple Endocrine Neoplasia*, Stockholm, Sweden 1994.
8. Borst MJ, Van Camp JM, Peacock ML, Decker RA. Mutational analysis of multiple endocrine neoplasia type 2A associated with Hirschsprung's disease. *Surgery* 1995;117:386-91.
9. Ceccherini I, Romei C, Barone V, Pacini F, Martino E, Loviselli A, Pinchera A, Romeo G. Identification of the cys 634 to tyr mutation of the RET proto-oncogene in a pedigree with multiple endocrine neoplasia type 2A and localised cutaneous lichen amyloidosis. *J Endocrinol Invest* 1994;17:201-4.
10. Edery P, Lyonnet S, Mulligan LM, Pelet A, Dow E, Abel L, Holder S, Nihoul-Fekete C, Ponder BA, Munnich A. Mutations of the RET proto-oncogene in Hirschsprung's disease. *Nature* 1994;367:378-80.
11. Romeo G Ronchetto P, Luo Y, Barone V, seri M, Ceccherini I, Pasini B, Bocciardi R, Lerone M et al. Point mutations affecting the tyrosine kinase domain of the RET proto-oncogene in Hirschsprung's disease. *Nature* 1994;367:377-8.

12. Mulligan LM, Marsh DJ, Robinson BG, et al. Genotype-phenotype correlation in multiple endocrine neoplasia type 2: report of the international RET mutation consortium. *J Int Med* 1995;238:343-6.
13. Donis-Keller H, Dou S, Chi D et al. Mutations in the RET proto-oncogene are associated with MEN 2A and FMTC. *Hum Mol Gen* 1993;2:851-6.
14. Mulligan LM, Eng C, Attie T, et al Diverse phenotypes associated with exon 10 mutations of the RET proto-oncogene. *Hum Mol Genet* 1994;3:2163-7.
15. Chew SL, Lavender P, Jain A, Weber A, Ross RJ, Wass JA, Besser GM, Clark AJ. Absence of mutations in the MEN 2A region of the RET proto-oncogene in non-MEN 2A pheochromocytomas. *Clin Endocrinol (Oxford)* 1995;42:17-21.
16. Lindor NM, Honchel R, Khosla S, Thibodeau SN. Mutations in the RET protooncogene in sporadic pheochromocytomas. *J Clin Endocrinol Metab* 1995;80:627-9.
17. Beldjord C, Desclaux-Arramond F, Raffin-Sanson M, Corvol JC, DeKeyser Y, Luton JP, Plouin JF, Bertagna X. The RET protooncogene in sporadic pheochromocytomas: frequent MEN 2-like mutations and new molecular defects. *J Clin Endocrinol Metab* 1995;80:2063-8.
18. Eng C, Smith DP, Mulligan LM, et al. Point mutation within the tyrosine kinase domain of the RET proto-oncogene in multiple endocrine neoplasia type 2B and related sporadic tumours. *Hum Mol Genet* 1994;3:237-41.
19. Gagel RF, Tashjian AH Jr, Cummings T, Papathanasoupoulos N, Kaplan MM, DeLillis RA, Wolfe HJ, Reichlin S. The clinical outcome of prospective screening for multiple endocrine neoplasia type 2A: an 18-year experience. *N Engl J Med* 1988;318:478-84.
20. Hamilton BP, Landsberg L, Levine RJ. Measurement of urinary epinephrine in screening for pheochromocytoma in multiple endocrine neoplasia type II. *Am J Med* 1978;65:1027-32.
21. Takai S, Miyauchi A, Matsumoto H et al. Multiple endocrine neoplasia type 2 syndromes in Japan. *Henry Ford Hosp Med J* 1984;32:246-50.
22. Gagel RF, Melvin KE, Tashjian AH Jr, Miller HH, Feldman ZT, Wolfe HJ, DeLillis RA, Cerviskinner S, Reichlin S. Natural history of the familial medullary thyroid carcinoma-pheochromocytoma syndrome and the identification of preneoplastic stages by screening studies: a five year report. *Trans Assoc Am Physicians* 1975;88:177-91.

23. Telenius-Berg M, Adolfsson L, Berg B et al. Catecholamine release after physical exercise: a new provocative test for early diagnosis of pheochromocytoma in multiple endocrine neoplasia type 2. *Acta Med Scand* 1987;222:351-9.
24. Neumann HPH, Wiestler OD. Clustering of features of von Hippel-Lindau syndrome: evidence for a complex genetic locus. *Lancet* 1991;337:1051-4.
25. Neumann HPH, Berger DP, Sigmund G et al. Pheochromocytomas, multiple endocrine neoplasia type 2, and von Hippel-Lindau disease. *N Engl J Med* 1993;329:1531-8.
26. Chen F, Kishida T, Yao M et al. Germline mutations in the von Hippel Lindau disease tumor suppressor gene: correlations with phenotype. *Hum Mutat* 1995;5:66-75.
27. Crossey PA, Richards FM, Foster K, et al. Identification of intragenic mutations in the von Hippel-Lindau disease tumour suppressor gene and correlation with phenotype. *Hum Mol Genet* 1994;3:1303-8.
28. Friedrich CA. Von Hippel-Lindau syndrome: a pleomorphic condition. *Cancer* 1999;86:S11:2478-82.
29. Zeiger MA, Zbar B, Keiser H, et al. Loss of heterozygosity on the short arm of chromosome 3 in sporadic, von Hippel-Lindau disease-associated, and familial phaeochromocytoma. *Genes Chromosomes Cancer* 1995;13:151-6.
30. Hull MT, Warfel KA, Muller J et al. Familial islet cell tumors in von Hippel-Lindau's disease. *Cancer* 1979;44:1523-6.
31. Carney JA, Go VLW, Gordon H, Northcutt RC, Pearse AG, Sheps SG. Familial pheochromocytoma and islet cell tumor of the pancreas. *Am J Med* 1980;68:515-21.
32. Mori Y, Kiyohara H, Miki T et al. Pheochromocytoma with prominent calcification and associated pancreatic islet cell tumor. *J Urol* 1977;118:843-4.
33. Nathan DM, Daniels GH, Ridgway EC. Gastrinoma and phaeochromocytoma: is there a mixed multiple endocrine adenoma syndrome? *Acta Endocrinol* 1980;93:91-3.
34. Zeller JR, Kauffman HM, Komorowski RA et al. Bilateral pheochromocytoma and islet cell adenoma of the pancreas. *Arch Surg* 1982;285:1618-9.
35. Gutmann DH, Cole JL, Stone WJ, et al. Loss of neurofibromin in adrenal gland tumors from patients with neurofibromatosis type 1. *Genes Chromosomes Cancer* 1994;10:55-8.

36. Gutmann DH, Geist RT, Rose K, et al. Loss of neurofibromatosis type 1 (NFL) gene expression in pheochromocytomas from patients without NFL. *Genes Chromosomes Cancer* 1995;13:104-9.
37. Dahia PLM, Grossman AB. The molecular pathogenesis of adrenal tumours. *Endocr Rel Cancer* 1995;2:267-79.
38. Kalff V, Shapiro B, Lloyd R, Sisson JC, Holland K, Nakajo M, Beierwaltes WH. The spectrum of pheochromocytoma in hypertensive patients with neurofibromatosis, *Arch Intern Med* 1982;142:2092-8.
39. Thakker RV. Molecular genetics of multiple endocrine neoplasia. Kohn Lecture of the Royal College of Pathologists. *Proc ACB Nat Meeting* 1997;2-3.

Catecholamines in urine after death

Abstract

Noradrenaline, adrenaline and dopamine levels were measured using HPLC and electrochemical detection and related to urine creatinine in 30 specimens from unselected autopsies. Values calculated from patients with essential hypertension were used as a reference and raised values of noradrenaline, adrenaline and dopamine were found in 76.6%, 80% and 23.3% respectively. Using the Mann-Whitney U test, there was no difference between the 11 patients with myocardial infarction and the 7 who died from severe head injury or multiple trauma. Physiological stress is common before death and urine noradrenaline values at autopsy overlap those found in some normal subjects and at autopsy in patients with phaeochromocytoma.

Introduction

Dying is likely to be stressful. Elevated catecholamines in urine and in vitreous have been suggested as an aid to diagnosis in death due to hypothermia¹. Elevations of phospholipid and catecholamine concentrations in the blood are increased in suffocation which may leave few or no morphological signs in the body². Increased urine noradrenaline has been reported in association with violent suicide attempts³.

With autopsy rates at <5% of hospital in-patient deaths in this institution, and with many patients relatives reluctant to agree to autopsy, urine catecholamines at the time of death may be relevant in discovering a phaeochromocytoma that may be suspected clinically, but may be difficult to find on imaging. In hypertensive patients, the triad of sweating attacks, tachycardia and headaches has a specificity of 93.8% and a sensitivity of 99.9% for phaeochromocytoma, but absence of these symptoms outrule the disease in 99.9%⁴. Thus a tumour may be strongly suspected clinically. In our series of 26 phaeochromocytomas and 1 paraganglioma, tumours were confirmed in 3 patients after death⁵. By using reference values derived from patients with essential hypertension which are higher than those from the normotensive population, raised values are more likely to reflect true stress⁶. This study reports representative urine catecholamine levels after death in an unselected series of autopsies and whether these levels are distinct from those found in phaeochromocytoma.

Methods

Urine was collected from 28 male and 2 female patients (no 13 and 17), aged from 18 to 83 with a median of 63 years who had urine present in the bladder at routine post mortem examinations. It was placed immediately into a urine container containing 0.5 ml of 6N HCl. The time from death was recorded and clinical details of the patients' final illness were noted. The specimens were centrifuged at 9,000 rpm for 5 min to remove debris. The analysis was performed on the supernatant. Urine free catecholamines were measured by high performance liquid chromatography with electrochemical detection⁷. The published method was modified to use a citrate mobile phase for the catecholamines

and a Biorad C18 column. The values were related to the urine creatinine measured by the Jaffe rate reaction on a Synchro CX7 (Beckman). The reference values were derived from 50 adult patients with essential hypertension.

Results

The median time from death to autopsy was 17 hours, ranging from 1 to 42 hours. The clinical details relating to the deaths and the catecholamine values are listed in Table 1. Raised values of noradrenaline, adrenaline and dopamine were found in 23 (76.6 %), 24 (80%), and 7 (23.3%) respectively. Patient 1 had taken beta-agonists for bronchodilatation and had received prolonged cardiopulmonary resuscitation. Patients with a final diagnosis of myocardial infarction were subdivided into those who were brought in dead into hospital and those who died following admission. Severe head injuries were routinely paralysed, ventilated and sedated but had to be drug free to fulfill the criteria for brain death. Raised urine catecholamine levels after death in these patients is surprising. Organs for transplantation were harvested from one patient (No 13). Those with multiple severe trauma had severe thoracic and abdominal injury following road traffic accidents. A comparison of the levels of noradrenaline and adrenaline in the 11 patients with myocardial infarction with the 7 patients with trauma using the two-tailed Mann-Whitney U test showed no significant differences; for noradrenaline $p = 0.82$ and for adrenaline $p = 0.256$. The patient with alcohol poisoning was found dead at home and the post-mortem alcohol levels were 304 mg per 100 ml in blood and 439 mg per 100 ml in urine. Patient number 20 was persistently confused following a road traffic accident and had bilateral frontal lobe contusions. He died from septicaemia from abscesses in the lungs and kidneys and he also had severe coronary atheromatous disease. Patient 22 had intractably bleeding varices and hepatocellular carcinoma having had a splenorenal shunt 30 years previously. The cause of death was unclear in the patient with trisomy 15 but cardiac arrhythmia was thought likely. Patient 24 died from food aspiration asphyxia. The electrocardiograph was flat on arrival but cardiopulmonary resuscitation (CPR) was unsuccessfully attempted during which three 3.0 mg bolus doses of adrenaline were administered. Similar CPR was also attempted on a young patient (no. 25) admitted pulseless following smoke inhalation from a house fire. Patient 26 died at home from acute hypovolaemia with colonic perforation. Patient 27 had the highest values. He was hypertensive and died from raised intracranial pressure and tonsillar herniation following a cerebellar haemorrhage. Both patients 27 and 28 died while on a morphine pump. Two patients (29 and 30) were brought in dead from respiratory failure. The data show overlap between the clinical categories and indicate that death is often stressful.

Final diagnoses with catecholamine levels in table 1 below. Units are mmol/mol creatinine. Patients number 13 and 17 were female. All others were male.

Table 1

Reference Values	Patient	Noradrenaline <0.04	Adrenaline <0.01	Dopamin <0.21
Myocardial Infarction in hospital	1	0.34	0.09	0.42
	2	0.24	0.064	0.28
	3	0.11	0.006	0.08
	4	0.07	0.016	0.04
	5	0.04	0.01	0.06
	6	0.03	0.006	0.1
M.I. - Brought in Dead	7	0.11	0.049	0.26
	8	0.1	0.026	0.14
	9	0.07	0.012	0.1
	10	0.04	0.007	0.1
	11	0.04	0.011	0.09
Severe Head Injuries	12	0.21	0.022	0.08
	13	0.15	0.045	0.17
	14	0.07	0.08	0.02
	15	0.01	0.177	0.07
Multiple Severe Trauma	16	0.12	0.024	0.27
	17	0.03	0.018	0.08
	18	0.16	0.005	0.13
Alcohol Poisoning	19	0.18	0.031	0.5
Septicaemia	20	0.16	0.127	0.17
Lung Cancer	21	0.08	0.012	0.24
Bleeding Varices	22	0.08	0.028	0.1
Trisomy 15	23	0.01	0.023	0.13
Asphyxia	24	0.03	0.007	0.13
Contd/...				
Smoke inhalation	25	0.05	0.013	0.05
Perforated Colon/hypovolaemia	26	0.04	0.016	0.1
Cerebral haemorrhage	27	1.22	0.189	1.21

Motor neuron disease	28	0.03	0.007	0.06
Chronic obstructive pulmonary disease	29	0.13	0.024	0.16
Asthma	30	0.02	0.005	0.07

Discussion

This study shows that raised catecholamine levels are common at the point of death. Our results are in contrast to a study of 119 medico-legal autopsy cases where the levels of serum catecholamines in instantaneous death were similar to the resting levels in humans whereas the levels following a prolonged death struggle corresponded to those found following maximal stress. However the levels of catecholamines in the individual case could not be used to indicate the length of the dying process. Urine values were not recorded⁸. The effect of autolysis in the bladder on postmortem catecholamines is unknown but there is little change in urine catecholamines stored at room temperature for 3 days within the urine pH range 0.5 to 7.0⁹. As there is no evidence that the patients were alkalotic before death, it is unlikely that there was substantial pH related change in the urine catecholamines during the interval between death and autopsy.

The highest noradrenaline value in this study is 1.22 mmol/mol creatinine. This lies intermediate between the urine noradrenaline values of 2.2 and 0.52 mmol/mol creatinine which we found in two patients in our series of phaeochromocytomas where excretion was related to creatinine^{5,10}. In overnight urine collections for phaeochromocytoma, Peaston et al reported noradrenaline values of 0.013 to 0.063 mmol/mol creatinine in non-tumour patients compared to values of 0.086 to 1.552 with phaeochromocytoma¹¹. Thus the overlap in values across different causes of death and various clinical circumstance limits the diagnostic value of post-mortem urine catecholamines.

The use of autopsy catecholamines may have a role in reviewing the effectiveness of differing modes of terminal care in the relief of anxiety and pain. Measuring urine catecholamine values before death may be a useful baseline in such a study but it is common to find an empty bladder at autopsy as urine frequently drains away as the sphincters relax.

References

1. Lapinlampi TO, Hirvonen JJ. Catecholamines in the vitreous fluid and urine of guinea pigs dying of cold and the effect of postmortem freezing and autolysis. *J Forensic Sci* 1986;31:1357-65.
2. Hirvonen J, Kortelainen ML, Huttunen P. Pulmonary and serum surfactant phospholipids and serum catecholamines in strangulation. An experimental study on rats. *Forensic Sci Int* 1997;90:17-24.
3. Mancini C, Brown GM. Urinary catecholamines and cortisol in parasuicide. *Psychiat Res* 1992;43:31-42.
4. Plouin P-F, Degoulet P, Tugaye A, Ducrocq M-B, Menard J. Le depistage du pheochromocytome: chez quels hypertendus? Etude semiologique chez 2585

hypertendus don't 11 ayant un pheochromocytome. *Nouv Presse Med* 1981;10:869-72.

5. O'Neill G, Tormey WP, Farrell MA, FitzGerald RJ, Finucane J. Phaeochromocytoma and lethal lactic acidemia. *Ir Med J* 1993;86:221.
6. Jones DH, Hamilton CA, Reid JL. Choice of control groups in the appraisal of sympathetic nervous activity in essential hypertension. *Clin Sci* 1979;57:339-44.
7. FitzGibbon M, FitzGerald RJ, Tormey WP, O'Meara A, Kenny D. Reference values for urinary HMMA, HVA, noradrenaline, adrenaline and dopamine excretion in children using random urine samples and HPLC with electrochemical detection. *Ann Clin Biochem* 1992;29:400-4.
8. Hausdorfer C, Pedal I, Zimmer G, Remppis A, Strobel G. Catecholamines, myofibrillary degeneration of the heart muscle and cardiac troponin T in various types of agony. *Arch Kriminol* 1995;196:46-57.
9. Miki K, Sudo A, Effect of urine pH, storage time, and temperature on stability of catecholamines, cortisol and creatinine. *Clin Chem* 1998;44:1759-62.
10. Tormey WP, FitzGerald RJ. Phaeochromocytoma- a laboratory experience. *Ir J Med Sci* 1995;164:142-5.
11. Peaston RT, Lennard TWJ, Lai LC. Can overnight excretion of urinary catecholamines and metabolites be used for the detection of phaeochromocytoma? *Proc ACB Nat Meeting* 1995;c93:172-3.

Clinical Investigation in Neuroblastoma

Neuroblastoma is an embryonal tumour, originating from cells of the sympathoadrenal lineage of the neural crest. It may arise anywhere in the sympathetic nervous system including the neck (3%), posterior mediastinum (20%), pelvis (3%), para-aortic paraspinal ganglia (24%) and the adrenal medulla (50%)¹.

Neuroblastoma is the commonest solid tumour in childhood . It accounts for 8% to 10% of all incident childhood cancers and 13% of cancer deaths in children in the UK. It occurs in about 1 in 7,000 to 10,000 live births.

Neuroblastomas may secrete catecholamines and their metabolites HVA and HMMA, 3-methyltyrosine, metanephrines, ferritin, vasoactive intestinal peptide (VIP), neuron specific enolase, lactic dehydrogenase (LDH) and acetylcholine. Hypertension occurs in 20% to 35% due to release of catecholamines.

Neuroblastoma is more common in children with Beckwith-Weideman syndrome (autosomal dominant with variable expression of exomphalos, macroglossia, gigantism, visceromegaly, renal medullary dysplasia and adrenocortical cytomegaly), neurofibromatosis, neuroblastosis, fetal phenytoin syndrome, fetal alcohol syndrome and Hirschsprung's disease. Intra-uterine exposure to phenytoin, phenobarbitone or ethanol may be environmental risk factors.

It is a heterogeneous disease with at least three distinct clinical and biological courses^{2,3}.

1. Lethal tumour progression
2. Maturation to ganglioneuroblastoma or ganglioneuroma – Ganglioneuroblastomas (malignant ganglioneuromas) show a degree of differentiation in between neuroblastoma and ganglioneuroma. Most are seen in young children but may occur in adults. They are usually found in the retroperitoneum or mediastinum. Tumour calcification is common. Ganglioneuroma is benign and usually seen in adults and are most commonly seen in the posterior mediastinum and retroperitoneum.
3. Spontaneous regression. This is a common feature of neuroblastoma and may occur with Stage IV-S tumours (Stages definitions to follow). Changes in the genes involved in apoptosis (bcl-2) and nerve growth factors may be critical. The theory has recently been discussed and its possible mechanisms hypothesised⁴.

Tumour Regression

Regression is a common occurrence in foetal development. The adrenal gland presents at week 12 and is the same size as the kidney, but it regresses during fetal and infantile development until it is a small gland. Similarly, the Organ of Zuckerkandl's ganglion at the origin of the superior mesenteric artery is a large paired organ at gestational week 8. The cells of embryonal paraganglia resemble and appear indistinguishable from the

neuroblast of a neuroblastoma. Spontaneous regression of stage IVs neuroblastoma is analogous to the fetal regression of Zuckerkandl and other paraganglia⁵. Beckwith and Perrin⁶ first described microscopic cell rests of neuroblastic nodules, “in situ neuroblastoma” in some infants dying of other diseases. It has been reported that virtually all fetuses have such foci, but it is still unclear whether these cell rests are the site of origin of malignant neuroblastomas⁷. It is clear that neuroblastoma has the highest rate of spontaneous regression of all human malignant neoplasms, but has one of the poorest outcomes when it occurs as disseminated disease in children. Regression in stage IVs without treatment is remarkable and important clinically^{8,9}. Screening studies from Japan and Quebec underscore the frequency of regression^{10,11}. Spontaneous regression has also been observed with Stage I and II tumours detected by biochemical screening and then observed rather than surgically excised¹². It has also been reported in a number of cases presenting with bone metastases¹³.

Clinical Classifications

Evans et al in 1971 proposed the clinical staging system which is used in prognosis¹⁴. This was based on the study of 100 cases. The Evans classification is still widely used¹⁵.

- Stage 1. Tumours confined to the organ or structure of origin.
- Stage II. Tumours extending in continuity beyond the organ or structure of origin but not crossing the midline. Regional lymph nodes on the ipsilateral side may be involved.
- Stage III. Tumour extending in continuity beyond the midline. Regional lymph nodes bilaterally may be involved.
- Stage IV. Remote disease involving skeleton, parenchymatous organs, soft tissues, or distant lymph node groups.
- Stage IV-S. Tumours which would be stage I or II except for the presence of remote disease confined to one or more of the following sites: liver, skin, bone marrow (without radiographic evidence of bone metastases on complete skeletal survey).

Other classifications have been published but are not widely used^{16,17}.

A suggested surgical classification is very similar and allows a more detailed examination of prognosis¹⁸.

International staging system for Neuroblastoma

Stage 1. Localized tumour confined to the area of origin; complete gross excision, with or without microscopic residual disease; identifiable same side and opposite side lymph nodes negative microscopically.

Stage 2A Unilateral tumour with incomplete gross excision; identifiable same and opposite side lymph nodes negative microscopically.

Stage 2B Unilateral tumour with complete or incomplete gross excision; with positive ipsilateral regional lymph nodes; identifiable contralateral lymph nodes negative microscopically.

Stage 3 Tumour infiltrating across the midline with or without regional lymph node involvement; or unilateral tumour with contralateral regional lymph node involvement; or midline tumour with bilateral regional lymph node involvement.

Stage 4 Dissemination of tumour to distant lymph nodes, bones, bone marrow, liver, and/or other organs (except as defined in Stage 4S)

Stage 4S Localised primary tumour as defined in stages 1 or 2A, with dissemination limited to liver, skin, or bone marrow. * (<10% of nucleated marrow cells are tumour cells). The infant must be less than 1 year of age.

This classification system is being used more widely internationally but it remains to be proven that the discrimination of stages 2A versus 3, or stage 1 with adhering lymph nodes versus 2B, is really of prognostic significance. Berthold notes the problem of staging in some cases between stages IV and IVs. "Strict adherence to the definition would result in classifying larger primary tumors (> 2A/B) or lymph node involvement beyond regional as stage 4 with the implementation of cytotoxic therapy. Experience from several countries suggests that it is worthwhile waiting for spontaneous regression in those patients⁵.

Immunohistochemistry

Neuroblastoma cells express neuron specific enolase, neuro-filaments, neurofilament – 66/alpha-internexin, peripherin, chromogranin, synaptophysin, secretogranin II, vasoactive intestinal peptide, micro-tubule-associated proteins, cell surface ganglioside GD₂. These together with electron microscopy are useful in distinguishing neuroblastoma from tumours such as rhabdomyosarcoma, Ewings Tumour/PNET and lymphoma¹⁹.

Prognosis

Prognosis depends on biological indices which seem more important than other factors including the clinical stage of the disorder and the age of the patient.

Biological factors in prognosis

Favourable – Triploid karyotype

High level of expression of TRK proto-oncogene.
High expression of *trk-A* receptor
No amplification of *N-myc* oncogene
No chromosome 1p abnormalities
Favourable Shimada tumour histology
More mature catecholamine metabolism
Normal neuron-specific enolase (NSE)
Normal ferritin

Unfavourable-pseudodiploid

Amplification of *N-myc* (more than 10 copies)
Expression of *bcl-2* gene
Low expression of *trk-A* receptor
Diploid DNA by flow cytometry
Unfavourable Shimada* tumour histology
Absence of S-100 protein positive cells in undifferentiated neuroblastoma.
Raised catecholamine synthesis and excretion
Elevated HVA:HMMA ratio
Higher NSE
Raised serum ferritin
Raised serum chromogranin A
Raised Creatine Kinase BB
Malnutrition at diagnosis
Urinary HMMA/HVA ratio <1
High telomerase activity

- Shimada and colleagues developed the conventional histopathological classification for neuroblastoma²⁰.

NSE may be used as tumour marker of patients with advanced disease. It may return to normal after therapy²¹. Increased ferritin levels, chromogranin A, and LDH may also be used as a tumour marker in neuroblastoma^{22,23}. Ganglioside GDs (II³ α (Neu Ac)₂GgOse₃Cer) is another putative circulating tumour marker for this disease²⁴.

Cells with functional Nerve Growth Factor (NGF) receptors may be susceptible to apoptosis leading to tumour regression or to differentiation leading to benign ganglioneuroma especially in older children. High levels of *N-myc* give tumours a growth advantage and may block the differentiation into mature cells or the onset of programmed cell death²⁵. The loss of heterozygosity on the short arm (p) of chromosome 1 precedes the development of amplification of *N-myc* oncogene^{26,27}. About 20% to 38% of neuroblastoma cases have deletions of 1p and this correlates closely with the clinical outcome. *N-myc* amplification can be detected by fluorescence in-situ hybridisation or

with the polymerase chain reaction (PCR). The N-*myc* protein product can be detected immunohistochemically²⁸. Mutations in p53 and *ras* genes are rarely found in neuroblastoma^{29,30}. Expression of the bcl-2 apoptosis suppressing protein is associated with unfavorable histology and N-*myc* amplification³¹. IGF-1 may play a role as IGF-1 receptor prevents the onset of apoptosis and favours tumour development³². Proteins involved in metastasis such as nm23 RNA and CD44 may also play a role^{33,34}.

Neuroblastoma tumour suppressor genes are found on chromosomes 1 and 17³⁵. Additional loci on 11q and 14q with loss of heterozygosity have also been reported³⁶.

A low urinary HMMA/HVA ratio is unfavorable because there is a relative deficiency of dopamine β -hydroxylase in the more primitive and aggressive tumours. The absence of a ganglioside Gt 1b indicates a poor prognosis³⁷.

About half of children with neuroblastoma present at a young age or at an early stage and these have a good prognosis despite minimal or no therapy. The tumour may be congenital. Biochemical screening of infants at less than 6 months of age does not seem to reduce the overall mortality because many tumours are discovered which will spontaneously regress. Infants with neuroblastoma usually have localised tumour with favourable biological characteristics and have an excellent prognosis even when there is advanced stage IV-S present

For those who present with neuroblastoma after the age of 1 year, they usually have advanced disease and a bad prognosis despite chemotherapy and bone-marrow transplantation^{2,3,38}. Expression of P-glycoprotein, a marker of multidrug resistance is a predictor of poor outcome of therapy³⁹. Prognosis is not affected by the patient's sex.

Breslow and McCann reported a 74% survival in children diagnosed before 11 months of age; a 26% survival when diagnosed between 12 and 23 months and 12% survival for those diagnosed at 24 months or older⁴⁰.

Survival is near 100% for stage I, 80% for stage II, 37% for stage III and 12% for stage IV.

Two year survival for the 57 patients presenting to Our Lady's Hospital for Sick Children in Dublin from 1979 to 1989 was 100% for Stage I (n=2), 86% for stage II (n=7), 55% for stage III (n=11), 12% for stage IV (n=33) and 50% for stage IVs (n=4)⁴¹. These are the results for the period prior to the commencement of the catecholamine service from Beaumont Hospital. This is an incidence of about 5 per year. Assuming the population in Ireland of about 800,000 in the paediatric age group, the Irish incidence is relatively low. However, some cases may not have been referred to the Dublin Centre and case ascertainment is likely to be incomplete.

Stage IVs

These tumours are biologically distinct and their clinical behaviour is remarkable. Katzenstein recently reported on 110 such patients⁴². Favourable prognostic features were usually present but diploidy was present in 18%, N-*myc* amplification in 9.5% and unfavourable histology in 10%. No unfavourable feature was present in 85 of 110 patients. Of the 25 patients with at least one bad prognostic factor, 8 died. The 3 year survival was 90% v 44% for N-*myc* amplification, 92% v 33% for good versus bad histology, and 68% v 90% for diploid versus hyperdiploid tumours. The average expression of the Trk A high affinity receptor for NGF was significantly higher in Stage IVs disease. One patient has been reported with high Trk A expression and N-*myc* amplification and their tumour regressed spontaneously⁴³. High telomerase activity is associated with poor outcome and advanced stage. Low or no detectable telomerase activity is a feature of Stage IVs⁴⁴. Thus infants with Stage IVs have excellent survival but there is a subset with a poor prognosis who can be identified by young age and unfavorable biological factors.

Whither biochemical screening for neuroblastoma?

Few if any tumours with poor-risk features are detected by infant screening programmes^{10,11}. It is probable that such tumours would continue to carry a poor prognosis even when discovered early.

Dr Woods succinctly set the question⁴⁵. “Can some favourable or mixed biology neuroblastomas progress to fatal, advanced-stage disease? If so, preclinical detection by screening should reduce disease-related mortality.”

In a large study of the 476,603 children born in Quebec from 1 May 1989 to 30 April 1994, HMMA and HVA were measured from filter paper samples taken from wet nappies using thin layer chromatography (TLC) as an initial screen. Borderline or positive TLC results were checked by gas chromatography/mass spectrometry. Samples were taken at 3 weeks and 6 months of age and compliance was 91% and 74% respectively. Woods and colleagues concluded that it is unlikely that screening in infants will reduce mortality from the disease¹¹.

Harm due to unnecessary intervention and stress to families may result from infant screening and Dr Bessho from Tokyo reviewed the evidence including that found in Japanese language papers in a ‘Lancet Commentary’ and concluded that “calling a halt to the (Japanese) national screening programme is a better option”⁴⁶.

False Negative Urinary VMA and HMMA

A further issue in screening is the finding in Quebec that, up to the age of 6 months, most children with neuroblastoma will not be detected using urinary HVA and HMMA. It has been assumed that all neuroblastomas are present in utero but this may not be true. Many

neuroblastomas do not continuously secrete catecholamines in large enough quantities for detection. The overall sensitivity of screening was 45%.

The European Neuroblastoma Study Group recorded all neuroblastomas or ganglioneuroblastomas from January 1982 and published their figures in 1989⁴⁷. This group has centres in Belgium, Ireland, UK, France, Greece, Holland, Italy and Norway. From a total of 619 patients, they reported that the percentages of patients with urine catecholamine metabolites (HMMA and HVA) >2 SD above the mean was 66% in Stage I, 63% in Stage II, 78% in Stage III, 90% in Stage IV and 98% in Stage IVs. These percentages were much the same in all centres. Classifying by age, the proportion secretors in Stage I was the same above and below the age of 1 year. For Stage II, 67% under 1 year and 55% over 1 year were secretors. The group concluded that screening of infants in Western Europe, by urine catecholamine metabolites alone will miss 30- 40% of Stages I and II tumours and about 20% of Stage III.

Incidence of neuroblastoma

Annual incidence ^{48,49}.

India	3.4 per 1,000000
New Zealand Maoris	13.7
UK (1971-80)	7.0
Germany (1982-84)	10.1
France (1975-85)	12.9

Age standardised incidence rates in children under 15 years old¹⁵.

UK -	10.1 per million
Germany -	11.4
Austria -	11.7
France -	12.5
Ireland (Republic)	5.5 (see below)

At Diagnosis: Stage IV disease ¹⁵ found in 43% in Denmark
41% in Quebec, Canada
45% in Delaware, USA.
62% in UK.
45% in France
39% in Germany
35% in Austria

The conclusion from the British figures is that diagnosis is delayed in the UK when compared to other developed countries.

Irish Figures

The latest available census of population figures are for 1996. These list a total of 859,424 children in the Republic of Ireland up to the age of 15 years. The comparable figure for 1991 is 940,574; for 1986 it is 1,024,701; for 1981 it is 1,043,729 and for 1979 it is 1,029,908.

Thus an average population figure for this age group for the years 1979 to 1989 can be taken as 1,032,779 from the census figures of 1979, 1981 and 1986. If the assumption is made that all neuroblastomas in childhood presenting clinically in the Republic of Ireland were seen at the Oncology Unit at Our Lady's Hospital for Sick Children in Dublin during that period, then the published figure of 57 patients over the 11 years from 1979 to 1989 gives an age standardised incidence under 15 years of age of about 5.02 per million per year. This is a low figure by international standards and case referral may have been less than complete. (see below for unreported adult case)

In the period December 1988 to August 1992 inclusive, ie 3.75 years, there were 21 patients reported in our study⁵⁰. That is 5.6 cases per year in a population of 940,574 giving an age standardized rate of 5.9 per million per year. It is noteworthy that one of the patients who had congenital undifferentiated neuroblastoma had all results within the reference range.

The Irish Tumour Registry began formal data collection in 1994. In that year, the incidence of neuroblastoma was 3, in 1995 there were 6. Taking the 1996 census figures, the age incidence rate up to 15 years is 4.5 per year, representing 5.23 per million. Again it is unlikely that all tumours are being notified to the tumour registry but the year by year figures are consistent and low.

Diagnosis

Plain X-Ray may reveal a mass often with stippled calcification. CT scan may define the boundaries of the tumour and may differentiate a neuroblastoma from a nephroblastoma. MRI is best for the detection of spinal involvement. ^{99m}Technetium scintiscan is useful in detecting bone metastases. To define the stage, to follow the course of the disease and to evaluate the response to treatment, imaging with MIBG is now considered essential⁵¹. Bone marrow may detect the presence of tumour rosettes. 24-hour collection for HMMA, HVA, dopamine and metanephrines usually confirms elevated levels in about 85% of patients. Base line blood counts and serum ferritin and NSE should be taken.

Adult neuroblastoma

Neuroblastoma is rare in adults. For example, only 39 cases (aged 16 to 75 years) had been reported up to 1986 in the world literature^{52,53}. Anatomical distribution is similar to that found in paediatric cases. The natural history of the disease may be longer and the tumour in adults may be less chemosensitive than that found in children. Median survival was 20.5 months with surgery and 12.5 months without. The detail of many of these

cases showed that in 14 of 27 cases, catecholamine excretion was not specified and it was raised in 9 of the 13 cases where measured. Stage IVs neuroblastoma is not seen in adults.

Histologically, neuroblastoma in children must be distinguished from Ewing's sarcoma, Wilm's Tumour and rhabdomyosarcoma. Whereas in adults, undifferentiated small cell carcinoma and lymphoma, granulosa cell tumour and rhabdomyosarcoma are the differential diagnosis.

There was one adult case in this hospital in the past 15 years to 1999. She was an 18 year old female who presented with left flank pain and had one of three urine cultures positive for E Coli. She subsequently had a left adrenal neuroblastoma which proved fatal.

Treatment

Stage 1 – Complete surgical excision.

Stage II – Complete surgical excision if histology is favourable, the tumour is aneuploid, there are <3 copies of N-*myc* oncogene, normal NSE and low ferritin levels. If some are indicating a poorer prognosis especially copies of N-*myc*, then chemotherapy should be added.

Stage III – Surgical resection alone may be appropriate if a total excision is possible and tumour markers are negative. If the tumour is large and resection difficult and unresectable, then a biopsy should be taken for histology, N-*myc* testing and DNA flow cytometry. Chemotherapy is used to shrink the tumour and then a re-evaluation is done to try for surgical excision of a shrunken tumour mass. If metastases are found then aggressive chemotherapy should be done.

Stage IV – Multiple drug cytotoxic chemotherapy with later tumour resection, regional radiation therapy for residual nonresectable retroperitoneal disease, total-body irradiation or near-lethal melphalan therapy and rescue with autologous bone marrow transplant in which marrow has been purged of potential neuroblasts with monoclonal antibodies.

Operation in Stage IV allows resection of the primary tumour and evaluation of the extent of lymph node involvement. It converts cases to complete response to treatment at a local site and determines if local irradiation is needed for an unresectable tumour.

Stage IVs – Treatment is controversial. Survival in these unusual cases with liver metastases, subcutaneous tumour nodules and sometimes bone marrow metastases with bone cortex invasion is greater than 80% even without treatment.

Patients with Stage IVs with bone marrow metastases and with more than 10 copies of N-*myc* should be treated with chemotherapy. Age and stage at diagnosis are the key to determining survival.

In association with Our Lady's Hospital for Sick Children in Crumlin, the usefulness of serial monitoring of urinary catecholamines, HMMA and HVA has been assessed as has the value of these markers in disease relapse⁵⁰. The study is described in detail in Chapter 21. Random urine samples were used in 21 patients and patients were monitored serially. The results were related to urine creatinine and where the results were on the reference value borderline, the procedure was repeated. Dopamine proved the most accurate indicator of persistent disease and noradrenaline and adrenaline were of little benefit as no patient showed an isolated elevation of either analyte. In this study, HVA was the analyte most often elevated. Nakawara et al has shown that patients presenting with neuroblastoma, who had been negative on mass screening programmes using HMMA and HVA only, were found to have elevated dopamine values +/- HMMA and or HVA at a subsequent diagnosis⁵⁴. These patients were also more likely to be in the poor prognosis group with *N-myc* amplification. These authors suggested that dopamine should be added to screening programmes especially in children over 1 year. Dopamine measurement should be included in the biochemical screen when neuroblastoma is suspected. However it should be considered that there are technical limitations to HPLC methods as a separate extraction procedure would be needed for dopamine by most methods.

Dopamine was found to be most informative in predicting the persistence of viable tumour despite therapy. We suggested that persistently elevated dopamine excretion is an indicator that targeted use of ¹³¹I MIBG would be appropriate in these cases. The German Neuroblastoma Trial found that in 47 children with relapsed, refractory or metastasising disease treated with MIBG, 9 children reached a complete or very good partial remission using the radio-isotope alone and another 13 reached a partial remission. Total dose was 283 +/- 203 mCi for stage III and 388 +/- 218 mCi for Stage IV⁵⁵.

Two of the 26 patients had normal biochemical values in all measured parameters despite the presence of microscopic residual disease at the time of surgery. In one patient with a thoracic recurrence, the biochemistry was also surprisingly normal.

In conclusion, normal values of HMMA, HVA and catecholamines in the presence of possible residual disease must not be interpreted as indicating that tumour is not present. Thus these parameters have serious limitations when used as tumour markers.

Novel Therapies

These include the use of differentiation inducer 13-cis-retinoic acid⁵⁶. Apoptosis inducers such as desferoxamine⁵⁷, anti-GD 2 monoclonal antibody⁵⁸ and cytokines, depletion of glutathione with buthionine sulfoximine⁵⁹ and fenretinide are also being studied^{60,61}.

References

1. Bolande RP. The neurocristopathies: a unifying concept of disease arising in neural crest maldevelopment. *Hum Pathol* 1974;5:409-29.

2. Woods WG, Lemieux B, Tuchman M. Neuroblastoma represents distinct clinical-biological entities: a review and perspective from the Quebec Neuroblastoma Screening Project. *Pediatrics* 1992;89:114-8.
3. Brodeur GM, Maris JM, Yamashiro DJ, Hogarty MD, White PS. Biology and genetics of human neuroblastomas. *J Pediat Hematol Oncol* 1997;19:93-101.
4. Pritchard J, Hickman JA. Why does stage 4s neuroblastoma regress spontaneously? *Lancet* 1994;344:869-70.
5. Berthold F. Neuroblastoma –commentary. *Eur J Cancer* 1997;33:1437-8.
6. Beckwith J, Perrin E. In situ neuroblastomas: a contribution to the natural history of neural crest tumors. *Am J Path* 1963;43:1089-104.
7. Ikeda Y, Lister J, Bouton JM, Buyukpamukcu M. Congenital neuroblastoma, neuroblastoma in situ and the normal fetal development of the adrenal. *J Ped Surg* 1981;16:636-44.
8. Haase D, Ablin AR, Miller C, Zoger S, Metthay KK. Complete pathologic maturation and regression of stage IVs neuroblastoma without treatment. *Cancer* 1988;62:818-25.
9. Matthay KK. Stage 4S neuroblastoma: what makes it special? *J Clin Oncol* 1998;16:2003-6.
10. Bessho F, Hashizume K, Nakajo T, Kamoshita S. Mass screening in Japan increased the detection of infants with neuroblastoma without a decrease in cases in older children. *J Pediatr* 1991;119:237-41.
11. Woods WG, Tuchman M, Robison LL, Bernstein M, Leclerc J-M, Brisson LC, Brossard J, Hill G, Shuster J, Luepker R, Byrne T, Weitzman S, Bunin G, Lemieux B. A population based study of the usefulness of screening for neuroblastoma *Lancet* 1996;348:869-70.
12. Yamamoto K, Hanada R, Kikuchi A et al. Spontaneous regression of localized neuroblastoma detected by mass screening. *J Clin Oncol* 1998;16:1265-9.
13. Sitarz A, Santulli T, Wigger H et al. Complete maturation of neuroblastoma with bone metastases in documented stages. *J Pediatr Surg* 1975;10:533-6.
14. Evans AE, D'Angio GJ, Randolph J. A proposed staging for children with neuroblastoma. *Cancer* 1971;27:374-8.

15. Powell JE, Esteve J, Mann JR, Parker L, Frappaz D, Michaelis J, Kerbl R, Mutz ID, Stiller C on behalf of SENCE. Neuroblastoma in Europe: differences in the pattern of disease in the UK. *Lancet* 1998;352:682-7.
16. Castleberry RP. Neuroblastoma. *Eur J Cancer* 1997;33:1430-8.
17. James DH Jr. Proposed classification of neuroblastoma. *J Pediatr* 1967;71:764
18. Smith EI, Haase GM, Seeger RC, Brodeur GM. Surgical perspective on the current staging in neuroblastoma: The international neuroblastoma staging system proposal. *J Pediatr Surg* 1989;24:386-90.
19. Oppedal BR, Brandtzaeg P, Kemshead T. Immunohistochemical differentiation of neuroblastomas from other small round cell neoplasms of childhood using a panel of mono- and polyclonal antibodies. *Histopathology* 1987;11:363-74.
20. Shimada H, Chatten J, Newton Jr WA. Histopathologic prognostic factors in neuroblastic tumors: definition of subtypes of ganglioneuroblastoma and age-linked classification of neuroblastomas. *J Nat Cancer Inst* 1984;73:405-13.
21. Zeltzer PM, Marangos PJ, Evans A, Schneider SL. Serum neuron-specific enolase children with neuroblastoma. *Cancer* 1986;57:1230-4.
22. Hann HL, Evans AE, Siegel SE, Wong KY, Sather H, Dalton A, Hammond D, Seeger RC. Prognostic importance of serum ferritin in patients with stages III and IV neuroblastoma: the children's cancer study group experience. *Cancer Res* 1985;45:2843-8.
23. Quinn JJ, Altman AJ, Frantz CN. Serum lactic dehydrogenase, an indicator of tumour activity in neuroblastoma. *J Pediatr* 1980;97:89-91.
24. Ladisch S, Wu Z-L. Detection of a tumor-associated ganglioside in plasma of patients with neuroblastoma. *Lancet* 1985;I:136-8.
25. Nakagawara A, Arima-Nakagawara M, Scavarda NJ, Azar CG, Cantor AB, Brodeur GM. Association between high levels of expression of the TRK gene and favorable outcome in human neuroblastoma. *N Engl J Med* 1993;328:847-54.
26. Brodeur GM. Neuroblastoma. Clinical significance of genetic abnormalities. *Cancer Surv* 1990;9:673-88.
27. Gilbert F, Feder M, Balaban G, Brangman D, Lurie DK, Podolsky R, Rinaldi V, Vinikoor N, Weisband J. Human neuroblastomas and abnormalities of chromosomes 1 and 17. *Cancer Res* 1984;44:5444-9.

28. Hiyama E, Hiya,a K, Yokoyama T, Ishii T. Immunohistochemical analysis of N-myc protein expression in neuroblastoma. Correlation with prognosis of patients. *J Pediatr Surg* 1991;26:838-43.
29. Komuro H, Hayashi Y, Kawamura M, Hayashi K, Kaneko Y, Kamoshita S, Hanada R, Yamamoto K, Hongo T, Yamada M et al. Mutations of the p53 gene are involved in Ewing's sarcoma but not in neuroblastoma. *Cancer Res* 1993;53:5284-8.
30. Moley JF, Brother MB, Wells SA, Spengler BA, Biedler JL, Brodeur GM. Low frequency of ras gene mutations in neuroblastomas, pheochromocytomas, and medullary thyroid cancers. *Cancer Res* 1991;51:1596-9.
31. Castle VP, Heidelberger KP, Bromberg J, Ou X, Dole M, Nunez G. Expression of the apoptosis-suppressing protein bcl-2, in neuroblastoma is associated with unfavorable histology and N-myc amplification. *Am J Pathol* 1993;143:1543-50.
32. Singleton JR, Randolph AE, Feldman EL. Insulin-like growth factor 1 receptor prevents apoptosis and enhances neuroblastoma tumorigenesis *Cancer Res* 1996;56:4522-9.
33. Leone A, Seeger RC, Hong CM, Hu YY, Arboleda MJ, Brodeur GM, Stram D, Slanon DJ, Steeg PS. Evidence for nm23 RNA overexpression, DNA amplification and mutation in aggressive childhood neuroblastomas. *Oncogene* 1993;8:855-65.
34. Comito MA, Savell VH, Cohen MB. CD44 expression in neuroblastoma and related tumors. *J Pediatr Hemato Oncol* 1997;19:292-6.
35. Plantaz D, Mohapatra G, Matthay KK, Pellarin M, Seeger RC, Feuerstein BG. Gain of chromosome 17 is the most frequent abnormality detected in neuroblastoma by comparative genomic hybridization. *Am J Pathol* 1997;150:81-89.
36. Srivatsan ES, Ying KL, Seeger RC. Detection of chromosome 11 and 14q sequences in neuroblastoma. *Genes Chromosomes Cancer* 1993;7:32-7.
37. Schengrund C-L, Repman MA, Shochat SJ. Ganglioside composition of human neuroblastomas. Correlation with prognosis. A pediatric oncology group study. *Cancer* 1985;56:2640-6.
38. Caitieberry RP. Biology and treatment of neuroblastoma. *Pediat Clin N Am* 1997;44:919-37.

39. Chan HS, Haddad G, Thorner PS, de Boer G, Lin YP, Ondrusek N, Yeger H, Ling V. P-glycoprotein expression as a predictor of the outcome of therapy for neuroblastoma. *N Engl J Med* 1991;325:1608-14.
40. Breslow N, McCann B. Statistical estimation of prognosis for children with neuroblastoma. *Cancer Res* 1971;31:2098-103.
41. Losty P, Quinn F, Breatnach F, O'Meara A, FitzGerald RJ. Neuroblastoma- a surgical perspective. *Eur J Surg Oncol* 1993;19:33-6.
42. Katzenstein HM, Bowman LC, Brodeur GM, Thorner PS, Joshi VV, Smith EI, Look AT, Rowe ST, Nash MB, Holbrook T, Alvarado C, Rao PV, Castleberry RP, Cohn SL. Prognostic significance of age, MYCN oncogene amplification, tumor cell ploidy, and histology in 110 infants with stage d(s) neuroblastoma: The Pediatric Oncology Group experience – A Pediatric Oncology Group study. *J Clin Oncol* 1998;16:2007-17.
43. Brodeur GM, Maris JM, Yamashiro DJ, Hogarty MD, White PS. Biology and genetics of human neuroblastomas. *J Pediatr Hematol Oncol* 1997;19:93-101.
44. Hiyama E, Hiyama K, Yokoyama T, Matsuura Y, Pietyszek MA, Shay JW. Correlating telomerase activity levels with human neuroblastoma outcomes. *Nat Med* 1995;1:249-55.
45. Woods WG. Unravelling mysteries of neuroblastomas. *Lancet* 1998;352:667-8.
46. Bessho F. Where should neuroblastoma mass screening go? *Lancet* 1996;348:1672.
47. Pritchard J, Barnes J, Germond S, Hartman O, De Kraker J, Lewis I, Lockwood L, Wallendszus for the European Neuroblastoma Study Group. *Lancet* 1989;ii:514-5.
48. Berthold, Hunneman DH, Kasewr H et al Neuroblastoma screening: argumentsnfrom retrospective analysis of three German neuroblastoma trials. *Am J Pediatr Hematol Oncol* 1991;13:8-13.
49. Parkin DM, Stiller CA, Draper GJ, Bieber CA, Terracini B, Young JL, eds. International incidence of childhood cancer. IARC Scientific Publications 87. Lyons IARC, 1988.
50. O'Meara A, Tormey W, FitzGerald RJ, FitzGibbon M, Kenny D. Interpretation of random urinary catecholamines and their metabolites in neuroblastoma. *Acta Paediatr* 1994;83:88-92.
51. Sisson JC, Shulkin BL. Nuclear medicine imaging of pheochromocytoma and neuroblastoma. *Q J Nucl Med* 1999;43:217-23.

52. Allan SG, Cornbleet MA, Carmichael J, Arnott SJ, Smyth JF. Adult neuroblastoma. Report of three cases and review of the literature. *Cancer* 1986;57:2419-21.
53. Kaye JA, Warhol MJ, Kretschmar C, Landsberg L, Frei E III. Neuroblastoma in adults. Three case reports and a review of the literature. *Cancer* 1986;58:1149-57.
54. Nakagawara A, Zaizen Y, Ikeda K, Suita S, Ohgami H, Nagahara N, Sera Y, Akiyama H, Kawakami K, Uchino J. Different genomic and metabolic patterns between mass screening-positive and mass screening-negative later presenting neuroblastomas. *Cancer* 1991;68:2037-44.
55. Klingebiel T, Berthold F, treuner J, Schwabe D, Fischer M, Feine U, Maul FD, Waters W, Wehinger H, Niethammer D. Metaiodobenzylguanidine (mIBG) in treatment of 47 patients with neuroblastoma: results of the German neuroblastoma trial. *Med Pediatr Oncol* 1991;19:84-8.
56. Finkelstein JZ, Krailo MD, Lenarsky C et al. 13-cis-retinoic acid (NSC 122758) in the treatment of children with metastatic neuroblastoma unresponsive to conventional chemotherapy: report from the Childrens Cancer Study Group. *Med Pediatr oncol* 1992;20:307-311.
57. Blatt J. Deferoxamine in children with recurrent neuroblastoma. *Anticancer res* 1994;14:2109-12.
58. Frost JD, Hank JA, Reaman GH, Friedrich S, Seeger RC, Gan J, Anderson PM, Ettinger LJ, Cairo MS, Blazar BR, Krailo MD, Matthay KK, Reisfeld RA, Sondel PM. A phase 1/1B trial of murine monoclonal anti-GD2 antibody 14.G2a plus interleukin-2 in children with refractory neuroblastoma. A report of the Children's Cancer Group. *Cancer* 1997;80:317-33.
59. Bailey H. Phase 1 clinical trial of intravenous L-buthionine sulfoximine and melphalan: an attempt at modulation of glutathione. *J Clin Oncol* 1994;12:194-205.
60. Mariotti A, Marcora E, Bunone G et al. N-(4-hydroxyphenyl)retinamide: a potent inducer of apoptosis in human neuroblastoma cells. *J Natl Cancer Inst* 1994;86:1245-7.
61. Ponzoni M, Bocca P, Chiesa V, Decensi A, Pistoia V, Raffaghello L, Rozzo C, Montaldo PG. Differential effects of N-(4-hydroxyphenyl)retinamide and retinoic acid on neuroblastoma cells: Apoptosis versus differentiation. *Cancer Res* 1995;55:853-61.

NEUROBLASTOMA – A BRIEF HISTORY

- 1803: Odier first used the term “neuroma”¹.
- 1847: Gunzberg reported a case of proliferating neuroma but Virchow did not consider the description sufficiently complete to warrant the diagnosis¹.
- 1864: Rudolf Virchow was the first to describe a neuroblastoma in a child with an abdominal tumour².
- 1870: Loretz reported the first well authenticated case of a ganglioneuroma³.
- 1879: Another ganglioneuroma described by Axel Key⁴.
- 1880: Marchant reported the first undifferentiated neuroblastoma⁵.
- 1881: Weichselbaum described a ganglioneuroma of the adrenal gland⁶.
- 1901: Beneke described a malignant transformation of a ganglioneuroma with metastases into the surrounding lymph glands⁷.
- 1901: Worcester described the first true neuroma of the central nervous system⁸.
- 1902: Kretz suggested that many of the combined round-cell sarcomas of the adrenal gland and the liver frequently reported in very young children are derived from formative sympathetic cells normally present in the foetus⁹.
- 1907: Falk pointed out that the tumours were nearly all in the sympathetic system and most occurred at less than 30 years of age¹⁰.
- 1908: Verocay described multiple neurofibromata¹¹.
- 1912: Pick and Bierchowski pointed out that the malignant neuroblastoma is the “unreifende” counterpart of the “reifende” ganglioneuroma and that these are part of the development of the sympathetic system and the adrenal gland¹².
- 1913: Landau characterised the tumours as (a) congenital, (b) appearance of malignancy early in childhood, (c) genetic relation to the neuroectoderm¹³.
- 1914: Wahl apparently found phaeochromocytomas in one case of neuroblastoma¹.
- 1934: Blacklock found small nests of tumour neuroblasts in his series of neuroblastomas which gave a weak chromaffin reaction¹⁴.
- 1957: An increased urinary excretion of pressor amines in neuroblastoma was first reported¹⁵.

- 1971: D'Angio, Evans and Koop defined the unique group with a specific metastatic pattern seen in infants and having a very high rate of spontaneous maturation and regression¹⁶. This is now called Stage IVs.
- 1983: Schwab et al discovered that human neuroblastoma cell lines have multiple copies of a DNA sequence that is related to the *v-myc* and *c-myc* oncogenes and designated it N-myc¹⁷.
- 1983: Emery, Shields, Shah and Garbes¹⁸ reported the first case of neuroblastoma associated with Beckwith-Wiedemann syndrome. These patients present with omphalocele, macroglossia, visceromegaly and hypoglycaemia. 10% have associated malignancies and there are 32 associated clinical characteristics and they are listed in this report.
- 1985: Seeger et al¹⁹ showed that genomic amplification of N-myc has a key role in determining the aggressiveness of neuroblastomas.

References

1. Wahl HR. Neuroblastoma: with a study of a case illustrating the three types that arise from the sympathetic system. J Med Res 1914;30:205-60.
2. Virchow R. Hyperplasie der Zirbel und der Nebenniere. In Hirschwald A. die krankhaften Geschwulste Vol. 2. Berlin, 18. Vorlesung, 7.2, 1963;148-150.
3. Loretz. Archiv fur Virchow 1870;xlix:435.
4. Key A. Virchow's Hisches Jahresb 1881;I:299.
5. Marchant f. Ueber eine eigenthumliche erkrankung des sympathicus der nebennieren und der peripherischen nerven (ohne bronzehaut). Arch f. Path Anat (Berlin). 1880,lxxxi:477-502.
6. Weichselbaum. Arch f Virchow 1881;lxxxv:54.
7. Beneke. Zeigler's Beitrage 1901:30.
8. Worcester. J Med Res 1901;I:59.
9. Kretz. Ergebn d Path 1902;8:532.
10. Falk. Beitrage f Zeigler. xl
11. Verocay Beitrage f Zeigler 1908;xlvi:403.
12. Pick, Bierchowski. Berl Klin Wochenschr 1913;1 and 2:16.

13. Landau. Frankfurter Zeitschr f Path 1913;11:1.
14. Blacklock JWS. J Path Bact 1934;39:27.
15. Mason GA, Hart-Mercer J, Millar EJ, Strang LB, Wynne NA. Adrenaline-secreting neuroblastoma in an infant. Lancet 1957;ii:322-25.
16. D'Angio G, Evans A, Koop C. Special pattern of widespread neuroblastoma with a favourable prognosis. Lancet 1971;i:1046-1049.
17. Schwab M, Alitalo K, Klempnauer K-H et al. Amplified DNA with limited homology to *myc* cellular oncogene is shared by human neuroblastoma cell lines and a neuroblastoma tumour. Nature 1983;305:245-8.
18. Emery LG, Shields M, Shah NR, Garbes A. Neuroblastoma associated with Beckwith-Wiedemann Syndrome. Cancer 1983;52:176-179.
19. Seeger RC, Brodeur GM, Sather H, Dalton A, Siegel SE, Wong KY, Hammond D. Association of multiple copies of the N-*myc* oncogene with rapid progression of neuroblastomas. N Engl J Med 1985;313:1111-6.

Reference values of catecholamines, HMMA and HVA excretion in children related to the urine creatinine.

In children, the urinary excretion of biogenic amines and their metabolites increases with age¹ but when these values are related to the urinary creatinine excretion, the relative values decrease^{1,2}. Age related reference values are especially important in the diagnosis of tumours such as neuroblastomas as 75% occur before the age of 4 years. The validity and convenience of random urine samples for these analytes has been accepted³⁻⁵. Urine creatinine is directly related to total muscle mass and is produced at a constant rate⁶. The serum creatinine is the best easily measured index of the glomerular filtration rate. Relating excretion to urinary creatinine minimizes the differences in children of similar ages due to size or sex, although this is controversial⁷. Taking daytime samples minimizes the influence of diurnal variation¹.

In neuroectodermal tumours, the pattern of urinary excretion of amines and their metabolites varies widely and dopamine as well as the adrenaline and noradrenaline pathways should be assessed for maximum sensitivity^{8,9}. Screening of infants with HMMA and HVA alone will miss 30 – 40% of stage 1 and 11 tumours and about 20% of stage 111 tumours¹⁰. The widely differing published reference values for the excretion of catecholamines and their metabolites reflect the diversity of analytical methods and data manipulations used. As noted in 1992, it still remains true in 1999, that the reports from the British external quality assessment scheme (UKEQAS) for urinary catecholamines confirm the continuing large variation in laboratory analytical performance. Screening programmes for neuroblastoma require the establishment of detailed age related reference ranges.

Materials and methods

Random urine samples were collected from 305 well children. These included 198 attending orthopaedic outpatients clinics, 44 from a general practice age register, 33 from neonatal and six week post-partum routine hospital baby checks and 30 from a daycare nursery. All specimens were collected during the normal working day. The urines were collected into universal containers pretreated with 0.5 ml 6N HCL except in neonates and in those children who were not toilet trained where disposable paediatric urine container bags were used. These were not pre-acidified but the urines were transferred to an acidified container immediately after collection. Samples were stored at -20° C and analysed in batches.

The methods were as set out in chapter 4. The alumina extraction method was used for the catecholamines. For HMMA and HVA, the sample volume was reduced from 1ml spiked with 100µl of standard to 0.5 ml spiked with 50 µl of standard to enable assay of small samples. Low sample volumes or technical difficulties were responsible for the differences in the number of results in some age groups. For example, shortage of sample prevented dilution and reanalysis of some concentrated urines with high dopamine values which had exceeded the detector scale. Some low adrenaline values were lost because of deterioration in the sensitivity of the assay for this analyte as indicted by internal control procedures. This was partly related to the recycling of the mobile phase, necessary due to cost.

Samples for urinary creatinine were diluted 1 in 10 and analysed on a Hitachi 704 using the Jaffe reaction without deproteinization. Since 1994, the Beckman CX7 was used and there

was a specific urine mode available. The method was again the Jaffe reaction with alkaline picrate. From the end of 1999, the Olympus 640 will be used with the same method principle.

Results

These are shown in figures 1 to 5 for each group with reference to the urine creatinine and are not normally distributed. Obtaining sufficient numbers of samples for statistical calculation of reference limits is a particular problem with children especially when stratification into several age groups is necessary. For example, at least 120 values are needed for reliable estimation of the 0.975 fractile¹¹. Since none of the age groups in this study reached this number, the suggested upper limits are based on the range of data values after elimination of outliers by visual inspection of the distributions and are displayed in figures 1 to 5. The broken line border represents the suggested upper limits and the extreme values would also have been excluded by the Dixon range statistic¹¹. A possible outlier is rejected if the distance between it and its nearest neighbour is greater than one-third of the range. Despite an apparent age-related trend in urinary adrenaline excretion, the scatter of values is such that we suggest that an arbitrary upper limit of 0.08 mmol adrenaline /mol creatinine be used but clearly further refinement of this limit may be beneficial.

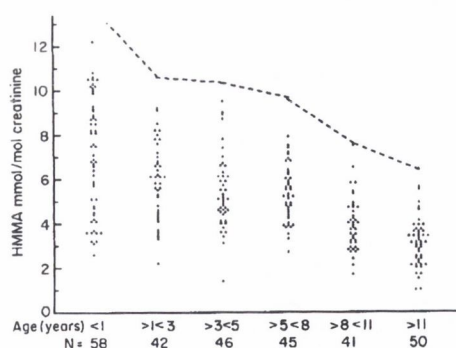


FIGURE 1. Age-related HMMA/creatinine ratio.
--- Suggested upper reference limit.

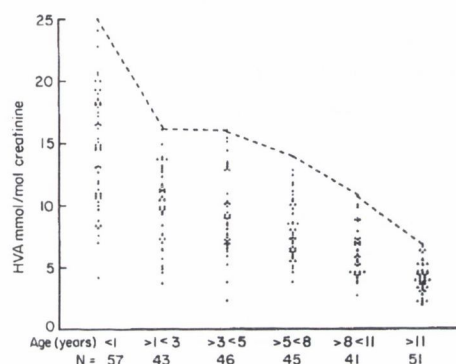


FIGURE 2. Age-related HVA/creatinine ratio.
--- Suggested upper reference limit.

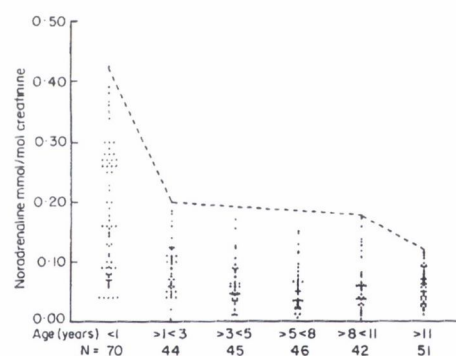


FIGURE 3. Age-related noradrenaline/creatinine ratio.
--- Suggested upper reference limit.

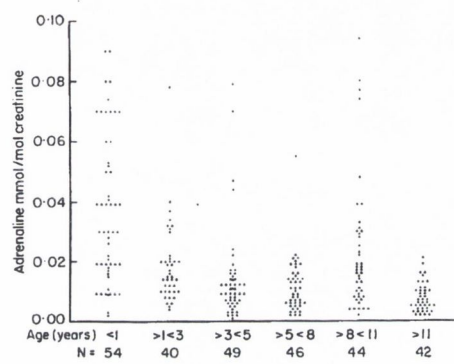


FIGURE 4. Age-related adrenaline/creatinine ratio.

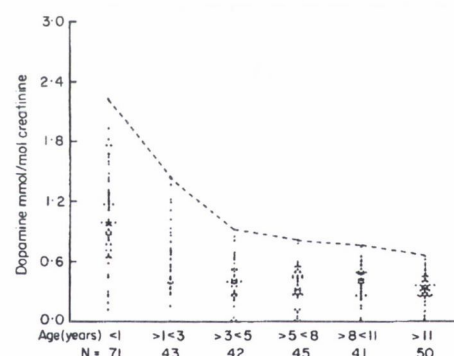


FIGURE 5. Age-related dopamine/creatinine ratio.
--- Suggested upper reference limit.

Age/Years	Number Of Subjects	HMMA	HVA
<1	58	<13.9	<25.0
1 – 3	42	<11.0	<17.0
3 – 5	46	<10.5	<16.0
5 – 8	45	<10.0	<14.0
8 – 11	41	< 7.5	<11.5
>11	50	< 7.0	< 7.0

Units in mmol/mol creatinine.

Age/Years	Number Of Subjects	Noradrenaline	Adrenaline	Dopamine
<1	70	< 0.430	< 0.080	< 1.95
1 – 3	44	< 0.200	< 0.080	< 1.45
3 – 5	45	< 0.190	< 0.080	< 0.95
5 – 8	46	< 0.180	< 0.080	< 0.85
8 – 11	42	< 0.170	< 0.080	< 0.75
> 11	51	< 0.130	< 0.080	< 0.65

Units in mmol/mol creatinine.

The above tabulated values are the working levels derived from figures 1 – 5 above.

Discussion

The importance of establishing reliable reference ranges for these analytes is highlighted by the possible improvement in prognosis for neuroblastoma with early detection¹². Walters et al² collated previously published reference ranges for HMMA excretion in children by Gitlow et al¹³ Haymond et al³ and Tuchman et al⁴. The methods used were either two-dimensional chromatography¹³, spectrophotometry³ or gas liquid chromatography⁴. Worthington et al¹⁴ and Haymond et al using the Pisano method¹⁵, or a modification quoted upper reference limits for HMMA which were widely divergent. The upper limits of the former approximate to the mean values of the latter study. Tuchman et al using GLC reported a broadly similar range to Haymond et al but with very low numbers of subjects in each group. The reference value subjects in the study by Worthington et al 1988 were ill inpatients who would be expected to be stressed whereas Haymond et al 1978 used healthy children only. The values here for HMMA are higher than but broadly mirror those of Worthington et al. The values published by Walters et al 1989 are similar to, though only slightly higher than, ours after the age of 1 year but are much higher before that. In the less than 1 year age group, our values were more in keeping with Gitlow et al. The reason for this is not apparent. The difficulties that we experienced in trying to collect 24 hr urines in very young children prompted us to use random specimens only whereas Worthington et al used 24 hour urine collections. Walters et al² used both and found a similar range of values whether 24 hour or random urine collections were used.

Soldin and Hill listed HMMA and HVA values on random urine samples from birth to 20 years¹⁶. Their ranges from children less than 1 year old were very high but the 100th percentile values for the rests of their groups were broadly similar to ours. Another paper, using HPLC, studied children of 3 years and older and reported HMMA and HVA values about 50% lower than reported here¹⁷. In their discussion, the authors acknowledged that younger children should have been studied because of the age incidence of neuroblastoma. Internal standard was omitted in their HMMA method but not in the method for catecholamines. Dale et al used gas chromatography with flame ionization detection and filter paper samples suitable for mass screening¹⁸. Data on 808 infants aged 6 months were log transformed to the normal distribution because of a slight skew and the transformed mean HMMA value was 6.8 µmol/mmol creatinine with an upper 95% confidence interval limit of 15 µmol/mmol. The mean HVA value was 10.9µmol/mmol creatinine with an upper 95% limit of 25.5 µmol/mmol. The HVA values were similar to here but the HMMA values were about 50% higher. Their method was reported linear to 16.0 µmol/L for HMMA and HVA

which is adequate for that age group. By contrast Walters et al² found a skewed distribution of values which were not normalized by logarithmic or square root transformation. They reported their data in a similar manner to that used here.

Describing the previously published data on catecholamine excretion in healthy children as 'scanty and incomplete', Parra et al¹⁹ themselves using small numbers found that catecholamine excretion per gram of urinary creatinine decreased with age. Their values were considerably lower than ours and they used Gaussian distribution statistics ignoring the obvious skew in their data. They favoured expressing their results in relation to body surface area, like De Schaepdryver et al²⁰, but the latter found no consistent change in the excretion of catecholamines or their metabolites with increasing size. Using HPLC methods, Rosano²¹ also found that the excretion of adrenaline, noradrenaline and dopamine decreased with age and quoted reference values considerably lower than here but higher than those of Premel-Cabic et al¹⁷. Paediatric reference ranges for catecholamines based on 24 hour collections were determined by Moyer et al²² but were not related to urinary creatinine. The recently published data by Reed et al²³ using Gaussian statistics for adrenaline, noradrenaline and dopamine excretion in relation to urinary creatinine were respectively only about 10%, 25% and 50% of the range here. But Henderson et al²⁴ reported dopamine and HVA values very similar to ours with HMMA levels considerably higher only in their youngest subjects. However some of these babies were ill inpatients.

A large study of 10,000 urine samples from 6 month old babies in England, there were significant differences between the sexes for HVA adjusted for creatinine, but not for HMMA²⁵. We did not research this question.

Our performance in the UKEQAS for urinary catecholamines has been close to the method mean for each analyte. Thus, the reference values shown here may be used by any laboratory with good analytical methods which has shown satisfactory performance in external quality assessment schemes for catecholamines and their metabolites.

A comprehensive list of the published reference ranges for catecholamines, HMMA, HVA, metanephrine and normetanephrine in children was published from this hospital in 1994²⁶.

References

1. Odink J, Thissen JTNM, Zeegers JH, Schreurs WHP. Diurnal variations in, and age and sex dependency on, the excretion of free catecholamines, homovanillic acid, vanilmandelic acid and 5-hydroxyindole-3-acetic acid in urine of children and adults. *Biogenic Amines* 1986;3:257-65.
2. Walters EG, Proctor DG, Elder GH, Walters G. urinary excretion of HMMA in children *Ann Clin Biochem* 1989;26:44-8.
3. Haymond RE, Knight JA, Bills AC. Normal values for urinary 3-methoxy-4-hydroxymandelic acid (VMA) in children. *Clin Chem* 1978;24:1853-4.
4. TuchmanM, Morris CL, Ramnaraine ML, Bowers LD, Krivit W. Value of random urine homovanillic acid and vanillylmandelic acid levels in the diagnosis and management of patients with neuroblastoma. Comparison with 24 hour urine collections. *Pediatrics* 1985;75:324-8.

5. Murphy SB, Cohn SL, Craft AW, Woods WG, Sawada T, Castleberry RP et al. Do children benefit from mass screening for neuroblastoma? Consensus statement from the American Cancer Society Workshop on Neuroblastoma screening. *Lancet* 1991;337:344-6.
6. Morgan DB, Dillon S, Payne RB. The assessment of glomerular function: creatinine clearance or plasma creatinine. *Postgrad Med J* 1978;54:302-10.
7. Applegarth DA, Ross PM. The unsuitability of creatinine excretion as a basis for assessing the excretion of other metabolites by infants and children. *Clin Chim Acta* 1975;64:83-5.
8. Tippet PA, McEwan AJ, Ackery DM. A reevaluation of dopamine excretion in phaeochromocytoma. *Clin Endocrinol* 1986;25:401-10.
9. Christofides JA, Fry DE. Why assessment of dopamine should be included in routine screening for urinary metanephrines. *Ann Clin Biochem* 1988;25(Suppl):119s-21s.
10. Pritchard J Barnes J, Germond S, Hartman O, de Kraker J Lewis I et al. Stage and urinary catecholamine metabolite excretion in neuroblastoma. *Lancet* 1989;ii:514-15.
11. Solberg HE. Approved recommendations (1987) on the theory of reference values. Part 5. Statistical treatment of collected reference values. Determination of reference limits. *Clin Chim Acta* 1987;170:s13-s32.
12. Nishi M, Miyake H, Takeda T, Shimada M, Takasugi N, Sato Y et al. Effects of mass screening of neuroblastoma in Sapporo City. *Cancer* 1987;60:43-6.
13. Gitlow SE, Mendlowitz M, Wikl EK, Wilk S, Wolf RL, Bertani LM. Excretion of catecholamine catabolites by normal children. *J Lab Clin Med* 1968;72:612-20.
14. Worthington DJ, Hammond EM, Eldeeb BB, Green A, Addison GM, Morris Jones PH. Neuroblastoma – When are urinary catecholamines and their metabolites ‘Normal’? *Ann Clin Biochem* 1988;25:620-6.
15. Pisano JJ, Crout RJ, Abraham C. Determination of 3-methoxy-4-hydroxy-mandelic acid in urine. *Clin Chim Acta* 1962;7:285-91.
16. Soldin SJ, Hill JG. Liquid chromatographic analysis for urinary 4-hydroxy-3-methoxymandelic acid and 4-hydroxy-3-methoxyphenylacetic acid and its use in investigation of neural crest tumours. *Clin Chem* 1981;27:502-3.
17. Premel-Cabic A, Turcant A, Allain P. Normal reference intervals for free catecholamines and their acid metabolites in 24 hour urines for children, as determined by liquid chromatography with amperometric detection. *Clin Chem* 1986;32:1585-7.
18. Dale G, McGill AC, Seviour JA, Craft AW. Urinary excretion of HMMA and HVA in infants. *Ann Clin Biochem* 1988;25:233-6.

19. Parra A, Ramirez del Angel A, Cervantes C, Sanchez M. Urinary excretion of catecholamines in healthy subjects in relation to body growth. *Acta Endocrinol* 1980;94:546-51.
20. De Schaepdryver AF, Hooft C, Delbeke MJ, Van den Noortgaete M. Urinary catecholamines and metabolites in children. *J Pediatr* 1978;93:266-8.
21. Rosano TG. Liquid chromatographic evaluation of age-related changes in the urinary excretion of free catecholamines in pediatric patients. *Clin Chem* 1984;30:301-3.
22. Moyer TP, Jiang N, Tyce GM, Sheps SG. Analysis for urinary catecholamines by liquid chromatography with amperometric detection: methodology and clinical interpretation of results. *Clin Chem* 1979;25:256-63.
23. Reed P, Bu'Lock D, Jones A, Layton T. Reference ranges for free catecholamine/creatinine ratios in random urines from normal children aged 5 to 16 years. *Ann Clin Biochem* 1991;28:297-8.
24. Henderson MJ, Heney D, McGinlay JM, Lewis I, Bailey C. Measurement of dopamine, HVA and HMMA in untimed urine samples: establishment of age related reference data in children. *Ann Clin Biochem* 1992;29:162-7.
25. Cole M, Parker L, Craft AW, Bell S, Dale G, McGill AC, Serviou JA, Smith J. Creatinine related reference ranges for urinary homovanillic acid and vanillylmandelic acid at 6 months of age. *Arch Dis Child* 1993;68:376-8.
26. FitzGibbon MC, Tormey WP. Paediatric reference ranges for urinary catecholamines/metabolites and their relevance in neuroblastoma diagnosis. *Ann Clin Biochem* 1994;31:1-11.

Interpretation of random urinary catecholamines and their metabolites in neuroblastoma

Neuroblastoma (NBL), one of the commonest malignancies in the paediatric age group, continues to remain a challenge to paediatric oncologists. In the diagnosis of NBL, random urine sampling and measurement of urinary catecholamines (uCATS) and their metabolites by HPLC or gas chromatography has superseded 24hr collections¹ and the less precise methods, e.g. Pisano et al² or a modification thereof. It is now the accepted practice of major paediatric oncology centres and has formed the basis of mass screening programmes aimed at preclinical detection of disease^{3,4}. These have entailed estimation of 4-hydroxy-3-methoxymandelic acid (HMMA) and homovanillic acid (HVA) in random samples of urine in children less than one year of age. While one or both of these catecholamine metabolites will be evaluated in most children with neuroblastoma, up to 20% of patients with advanced disease will have normal values⁵. The aims of this study were: (a) to report experience in HPLC measurement of catecholamines and their metabolites in random urinary samples in the diagnosis of NBL, (b) to assess the usefulness of serial monitoring in predicting response to chemotherapy and (c) to assess the predictive value of uCATS / metabolites in disease relapse.

Patients

Twenty one patients who presented with neuroblastoma between December 1988 and August 1992 were included in the study. There were 14 males and 7 females. Age at diagnosis ranged from birth to 76 months (median 29 months). Patients were staged using the International Neuroblastoma Staging System⁶. One patient had stage 1 disease, 1 stage 2A, 6 stage 3, 12 stage 4 and 1 infant presented at one month of age with stage 4S disease. All patients were monitored serially after diagnosis.

Five patients had primary surgical resection (1 stage 1, 1 stage 2A, 2 stage 3 and 1 stage 4); the infant with stage 4S disease received no treatment and 1 stage 4 patient died within 48h of diagnosis. Patients with stage 3 and 4 disease were treated according to the European Neuroblastoma Study Group (ENSG)⁷ and other established⁸ protocols.

Five of the 21 patients developed recurrent disease, 2 – 24 months after completion of treatment.

Samples

Random urinary samples were collected into pre-acidified containers as described previously⁹ and results reported in relation to urinary creatinine. Urinary creatinine was estimated using a Jaffe kinetic method without deproteinisation on a Hitachi 704 autoanalyser with sample predilution. Reference values and methods have been reported previously⁹.

Results

A wide range of values was observed for most uCATS / metabolites. Repeat assays of borderline values confirmed the accuracy of original data with less than 5% variation noted in all cases.

Diagnosis

The uCAT/metabolite results of the 21 patients studied are reported in Table 1 and recorded graphically on a log scale in Fig 1. Twenty of these patients had elevation of one or more uCATs/metabolites at diagnosis: in 5 patients all analytes were raised, 3 patients had elevation of four, 5 patients had three, 4 patients two and in 3 patients just one metabolite was elevated, dopamine in 2 patients and HMMA in 1. All results were within the reference range in 1 patient (No. 6), with congenital undifferentiated NBL, using the classification of Joshi et al¹⁰.

Presurgical evaluation

Biochemical results for those patients who did not have primary resection (n = 13) are shown in Table 2. All of these patients had achieved at least very good partial remission after chemotherapy (i.e. greater than 90% reduction in primary tumour with reversion to normal bone scan and, in all but 1 patient, clearance of tumour from bone marrow). Elective resection was preformed 3-6 months after initiation of treatment. Two patients had completely normal profiles, despite the presence of microscopic residual disease at surgery. Conversely, 1 patient had no viable disease detected, yet dopamine was elevated immediately prior to surgery. Dopamine was also the only CAT/metabolite elevated in 5 other patients, all of whom had viable tumour resected.

Serial uCAT/metabolite estimations were particularly helpful in monitoring disease activity in 1 infant with stage 4 NBL (patient No 18 Tables 1,2) who had elective resection of a left adrenal primary six months after diagnosis. At that time, she still had persistence of multiple liver metastases, confirmed on biopsy. No further treatment was given. HMMA was elevated at the time of elective surgery but reverted to normal within four months of surgery; serial ultrasound examinations continued to show regression in the size of metastases, consistent with spontaneous regression of disease. Another infant with stage 4S disease showed spontaneous regression of disease with reversion of all uCATS/metabolites to normal by 14 months.

Relapse

Five of 21 patients developed recurrent disease within the study period (Table 3 and Fig 2). One of these patients (No 14) had persistently elevated dopamine levels from the time of surgery, although no tumour was detected at that time, and HVA also became elevated prior to the development of diffuse bone disease seven months after surgery. All other patients had reverted to normal values between completion of treatment and development of recurrent disease. All patients with diffuse bone disease had elevated HVA $\pm$ HMMA $\pm$ dopamine. One patient with recurrent disease in the posterior mediastinum had normal values for all analytes.

Table 1. Urinary CATs/metabolites at diagnosis.

Patient No.	Age at diagnosis (years)	Stage	HMMA	HVA	Dopamine	Noradrenaline	Adrenaline
1	2.8	4	246.0 (11.0) ^a	163.0 (17.0) ^a	8.5 (1.45) ^a	1.11 (0.20) ^a	0.55 ^b
2	2.7	4	5.6 (11.0)	16.5 (17.0)	3.75 (1.45)	0.06 (0.20)	0.05
3	2.4	2A	22.4 (11.0)	24.3 (17.0)	1.17 (1.45)	0.11 (0.20)	0.05
4	0.5	3	66.4 (14.0)	—	0.82 (1.95)	0.20 (0.43)	0.04
5	3.8	4	27.2 (10.5)	47.8 (16.0)	1.03 (0.95)	0.17 (0.19)	0.01
6	Birth	1	8.1 (14.0)	22.0 (25.0)	0.90 (1.95)	0.26 (0.43)	0.01
7	2.4	3	5.2 (11.0)	—	10.0 (1.45)	—	—
8	2.1	3	11.3 (11.0)	170.0 (17.0)	89.6 (1.45)	0.04 (0.20)	0.03
9	2.2	4	197.0 (11.0)	898.0 (17.0)	80.2 (1.45)	5.77 (0.20)	0.61
10	2	4	12.3 (11.0)	58.0 (17.0)	23.7 (1.45)	0.67 (0.20)	0.38
11	6.3	4	37.8 (10.0)	—	10.6 (0.85)	0.30 (0.18)	0.02
12	0.1	4S	363.0 (14.0)	291.0 (25.0)	1.53 (1.95)	0.49 (0.43)	0.34
13	1.6	3	45.6 (11.0)	43.3 (17.0)	0.82 (1.45)	0.07 (0.20)	0.14
14	1.4	4	18.4 (11.0)	343.7 (17.0)	—	1.60 (0.20)	—
15	5.3	4	14.3 (10.0)	83.0 (14.0)	54.7 (0.85)	1.04 (0.18)	0.36
16	4.25	3	156.7 (10.5)	363.7 (16.0)	8.69 (0.95)	0.47 (0.19)	0.43
17	5.75	4	241.0 (10.0)	108.0 (25.0)	0.20 (0.85)	0.07 (0.18)	0.02
18	0.5	4	582.0 (14.0)	299.0 (25.0)	2.77 (1.95)	0.19 (0.43)	1.64
19	4.3	3	6.3 (10.5)	24.0 (16.0)	1.03 (0.95)	0.05 (0.19)	—
20	2.5	4	170.0 (11.0)	159.0 (17.0)	1.47 (1.45)	0.24 (0.20)	0.06
21	0.9	4	12.6 (14.0)	46.8 (25.0)	18.5 (1.95)	0.08 (0.43)	0.05

^a Upper limit of normal for age; units reported in mmol/mol creatinine; ^b 0.08 upper limit of normal. — = Insufficient for assay.

90 A O'Meara et al.

ACTA PÆDIATR 83 (1994)

Urinary CATs/Metabolites at Diagnosis

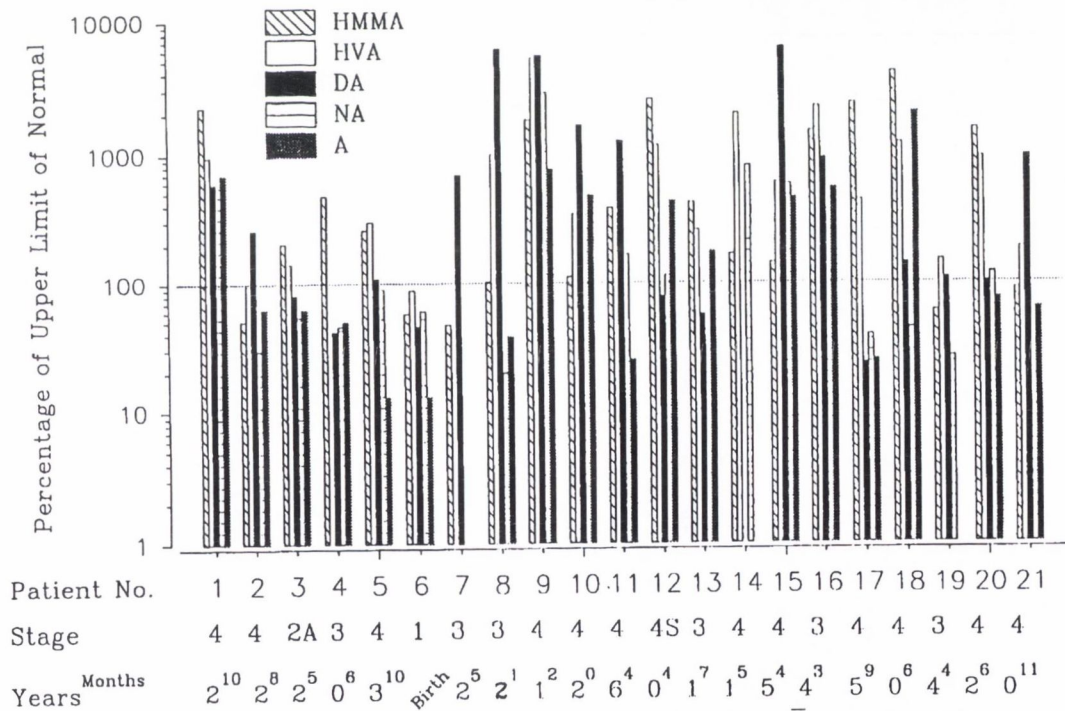


Fig. 1. Diagnostic profile of uCATs/metabolites in 21 patients with neuroblastoma. As the range of results for all uCATs/metabolites is very wide, all data are reported on a log scale and related to our upper limit of normal for age (see ref (9)); 100% = upper limit of normal for each value.

Table 2. Urinary CATs/metabolites at pre-surgical evaluation.

Patient No.	uCATs/metabolites	Tumour
1	All normal	Yes
4	HMMA↑; HVA↑; DA↑	Yes
5	All normal	Yes
7	DA↑	Yes
8	DA↑	Yes
10	HVA↑; DA↑	Yes
11	DA↑	Yes
14	DA↑	No
15	HMMA↑; HVA↑; DA↑; NA↑	— ^a
17	DA↑	Yes
18	HMMA↑	Yes
19	All normal	— ^b
21	DA↑	Yes

^a No surgery because of progressive disease; ^b no surgery because of complete tumour regression.

Table 3. Urinary CATs/metabolites at relapse.

Patient No.	uCATs/metabolites	Site of relapse
10	HMMA↑; HVA↑; DA↑	Diffuse bone
11	HMMA↑; HVA↑; DA↑	Diffuse bone
14	HVA↑; DA↑	Diffuse bone
17	HMMA↑; HVA↑	Diffuse bone
19	All normal	Thorax

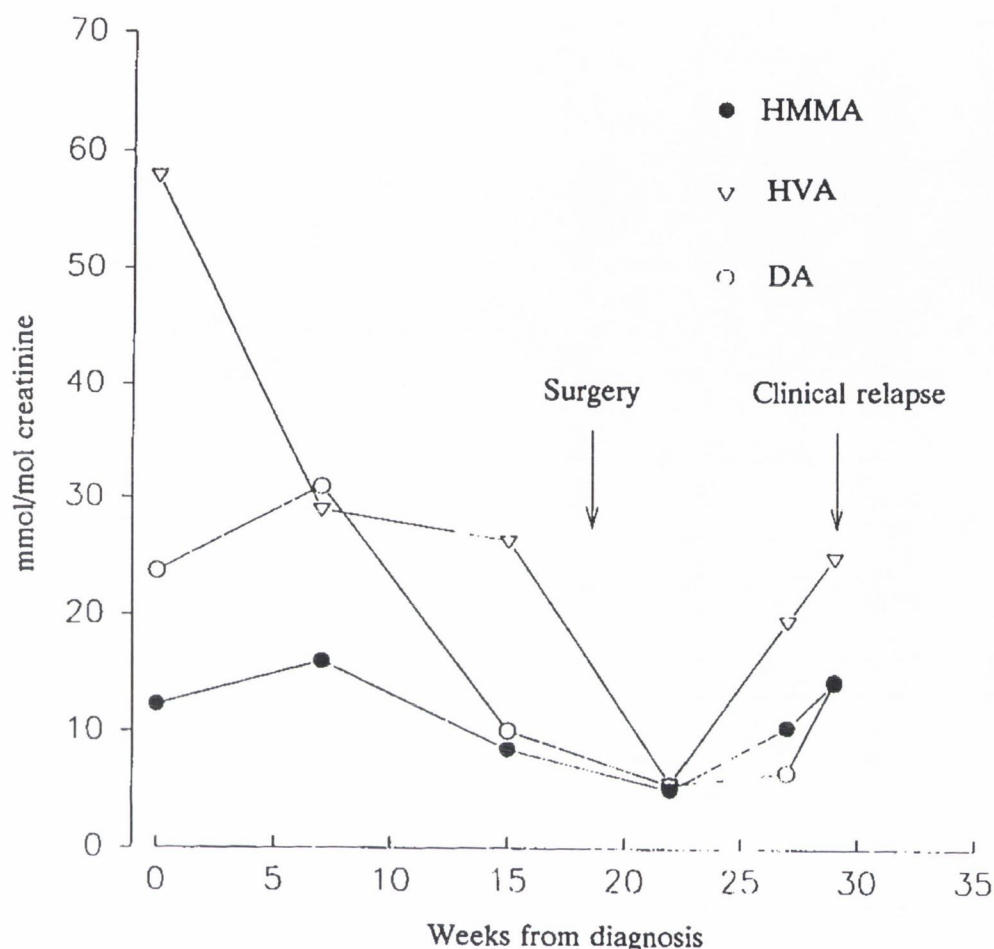


Fig. 2. Serial HMMA, HVA and dopamine (DA) values for one patient (No.10) who died of disease 32 weeks from diagnosis.

Discussion

This study has confirmed the reliability of HPLC measurement of CATs/metabolites in random urinary samples in the diagnosis of neuroblastoma. Caution should be exercised, however, when results are corrected for urinary creatinine, as excretion is altered by ingestion of meat¹¹. Acetoacetate may interfere positively, and some cephalosporins negatively, with the kinetic Jaffe creatinine method¹². In view of the fact that day-to-day analytical coefficient of variations of creatinine in urine or plasma may be in the range 5-7% and that there is an inherent intra-personal fluctuation in uCATs/metabolites, all borderline values should be repeated routinely.

In this study, HVA was the CAT/metabolite most often elevated. Pritchard et al⁵ noted that only 64% of patients registered by the ENSG with early stage disease and 87% patients with advanced disease had elevation of HMMA and/or HVA. Nakagawara et al¹³ have identified a subgroup of patients with adverse prognosis who had increased urinary dopamine excretion at

diagnosis and normal HMMA and HVA. A more recent study by his group demonstrated that patients presenting with NBL who had been negative on mass screening programmes (which screened for HMMA and HVA only) were found to have elevated dopamine values $\pm$ HMMA/HVA at a subsequent diagnosis¹⁴. These patients were also more likely to have N-myc amplified tumours and prognosis for this cohort was poor. These data are in keeping with the concept that tumours which do not possess a capacity to degrade CATs behave more aggressively¹⁵. Nakagawara's group concluded that dopamine should be added to HMMA and HVA in mass screening programmes, particularly in children older than one year. As measurement of dopamine by HPLC requires a separate extraction procedure for analysis, routine incorporation into screening programmes might prove time consuming, expensive and impractical. It would seem advisable, however, that dopamine be included when a diagnosis of NBL is suspected. No patient in this series showed an isolated elevation of noradrenaline or adrenaline.

UCAT/metabolite profiles at the pre-surgical evaluation merit special consideration; dopamine was most informative in predicting persistence of viable tumour, being elevated in 9 of 13 patients, 1 of whom had no detectable disease at the time of surgery but developed recurrent disease seven months later, despite high-dose melphelan and autologous bone marrow transplant. It is reasonable to assume that chemotherapy had eliminated the biochemically more mature cells, allowing the more immature chemoresistant dopamine-secreting cells to persist. Based on this premise, it would therefore seem appropriate that those patients with persistently elevated dopamine levels at elective resection would be selected for alternative treatment e.g. targeted therapy with ¹³¹I MIBG¹⁶. All values were normal in 2 patients who still had microscopic residual disease at the time of surgery, indicating their limitations as tumour markers.

All patients who developed diffuse bone disease at relapse had elevation of HVA $\pm$ HMMA $\pm$ dopamine. The finding of a normal uCAT/metabolite profile in 1 patient with recurrent intrathoracic disease was unexpected; this may have been due to the predominance of a biochemically inert tumour, as was also the case in 1 patient with congenital neuroblastoma. Clearly, normal uCATS/metabolites in the presence of suspicion of recurrent disease must be interpreted with caution.

In conclusion, this study adds to the existing evidence that random rather than 24hr samples of urine for measurement of HMMA, HVA and dopamine by HPLC can be used as reliable indicators in the diagnosis of NBL. The findings indicate that serial measurement is useful in assessing response to chemotherapy and that dopamine may be more reliable than HMMA or HVA in predicting persistence of disease: larger scale studies are needed to confirm this observation. The study demonstrates that catecholamine profiles may be within normal values at relapse and should be interpreted with caution, particularly with local/isolated lesions.

Measurement of noradrenaline and adrenaline in NBL would appear to be of little value, either at diagnosis or in subsequent monitoring of disease.

References

1. Tuchman M, Morris CL, Ramnaraine ML, Bowers LD, Krivit W. Value of random urinary HVA and VMA in the diagnosis and management of patients with neuroblastoma: comparison with 24 hour urine collections. *Pediatrics* 1985;75:324-8.

2. Pisano JJ, Crout RJ, Abraham C. Determination of 3-methoxy-4-hydroxymandelic acid in urine. *Clin Chim Acta* 1962;7:285-91.
3. Nishi M, Miyake H, Takeda T, Shimada M, et al. Effects of the mass screening of neuroblastoma in Sapporo City. *Cancer* 1987;60:433-6.
4. Woods WG et al. Neuroblastoma screening. 2nd International Symposium, Minnesota. 1991;30A.
5. Pritchard J et al (for ENSG). Stage and urinary catecholamine metabolite excretion in neuroblastoma. *Lancet* 1989;ii:514-15.
6. Brodeur GM, Seeger RC, Barrett A, Berthold F, Castleberry RP, De Bernardi B, Evans AE, Favrot M, Freeman AI, et al. International criteria for diagnosis, staging and response to treatment in patients with neuroblastoma. *J Clin Oncol* 1988;6:1974-81.
7. Shafford EA, Rogers DW, Pritchard J. Advanced neuroblastoma: improved response rate using a multiagent regimen (OPEC) including sequential cisplatin and VM-26. *J Clin Oncol* 1984;2:742-7.
8. Lockwood L, Lewis IJ, Eden O, Breatnach F. JOSEPHINE. Platinum based dose intensive induction regime for advanced stage neuroblastoma. *Med Pediatr Oncol* 1990;18:393A.
9. Fitzgibbon M, FitzGerald RJ, Tormey W, O'Meara A, Kenny D. Reference values for urinary HMMA, HVA, noradrenaline, adrenaline and dopamine excretion in children using random urine samples and HPLC with electrochemical detection. *Ann Clin Biochem* 1992;29:400-4.
10. Joshi VV, Cantor AB, Altshuler G, Larkin EW, Neill JS, Shuster J, Holbrook CT, Hayes FA, Castleberry RP. Recommendations for modification of terminology of neuroblastic tumors and prognostic significance of Shimada classification. A clinicopathologic study of 213 cases from the Pediatric Oncology Group. *Cancer* 1992;69:2183-96.
11. Jacobsen FK, Christensen CK, Mogensen CE, Heilskov NS. Evaluation of kidney function after meals. *Lancet* 1980;i:319.
12. Payne RB. Creatinine clearance: A redundant clinical investigation. *Ann Clin Biochem* 1986;23:243-50.
13. Nakagawara A, Ikeda K, Tasaka H. Dopaminergic neuroblastoma as a poor prognostic subgroup. *J Pediatr Surg* 1988;23:346-9.
14. Nakagawara A, Zaizen Y, Ikeda K, Suita S, Ohgami H, Nagahara Akiyama H, Kawakami K, Uchino J. Different genomic and metabolic patterns between mass screening-positive and mass screening-negative later presenting neuroblastomas. *Cancer* 1991;68:2037-44.

15. Laug WE, Siegel SE, Shaw KN, Landing B, Baptisita J, Gutenstein M. Initial urinary catecholamine metabolite concentrations and prognosis in neuroblastoma. *Pediatrics* 1978;62:77-83.
16. Klingebiel T, Berthold F, Treuner J, Schwabe D, Fischer M, Feine FD, Waters W, Wehinger H, Niethammer D. Metaiodobenzylguanidine (mIBG) in treatment of 47 patients with neuroblastoma: results of the German neuroblastoma trial. *Med Pediatr Oncol* 1991;19:84-8.

Concluding discussion

At the year 2000, the use of urine catecholamine screening remains as the best initial test in the diagnosis of phaeochromocytoma. Urinary free catecholamines (noradrenaline, adrenaline and dopamine) and or total metanephrine (normetanephrine and metanephrine) are the most sensitive. Expected sensitivities for either group are in the high 90%^s. This is a clear sensitivity benefit over HMMA (VMA) whose sensitivities are usually at least 10% less. Local practice usually dictates which tests are used. HPLC methods are most often used. The UKNEQAS for urinary catecholamines shows the overall state of current practice. The October 1999 distribution number 56 shows that noradrenaline was measured in 71 laboratories with 66 using HPLC and 63 of those using electrochemical detection. HMMA was measured in 40 laboratories with 31 using HPLC and 29 of these using electrochemical detection. Thus the methods used in the present studies are the current practice norms. As catecholamine values are higher in patients with hypertension than in normotensives, it is important that the reference intervals used are derived from hypertensive patients who do not have phaeochromocytoma. This keeps the number of false positives who require imaging within manageable levels while minimizing the number of false negatives. There was one patient in this study with false negative urine noradrenaline values. This patient's data had a number of values from 24 hour collections that were remarkably low. Of the nine collections, five (217, 242, 185, 447 and 383 nmol/24 hours) had noradrenaline values that would have constituted false positives even if calm normotensives had been used as the reference base population.

Phaeochromocytomas may be investigated as out-patients or in day wards. The patients' hypertension must be controlled by drugs to avoid catastrophe from lactic acidemia, cardiac arrhythmia, heart failure, myocardial infarction or stroke. Current first line antihypertensives such as diuretics, betablockers, ACE inhibitors, alpha blockers and calcium channel antagonists (except possibly nifedipine) do not interfere with the alumina extraction method used for this thesis. However, labetalol, alpha methyl dopa (aldomet), 'sinemet' and paracetamol cause interference with the alumina extraction method (see chapter 4). Aldomet remains a first line antihypertensive in obstetric practice. It does not interfere with the noradrenaline peak but it makes interpretation difficult. Nifedipine interference with the adrenaline peak may have been responsible for a number of false positive adrenaline screens as it is a commonly used drug. A policy of screening urines with high catecholamine values for drugs seems sensible to aid interpretation and the determination of clinical significance. In the year 2000, the automated HPLC 'REMEDI' analyser (Rapid, EMERgency Drug Identification, BioRad) which allows a very wide screen of over 900 drugs in serum and urine to be carried out easily is being brought into routine service at Beaumont Hospital¹. This will allow a systematic study of interferences to be carried out.

The conventional concept is that a phaeochromocytoma is simply a paraganglioma confined to the adrenal. These tumours have been lumped together historically by their histopathology. They produce catecholamines and other tumour markers in common. Yet genetic studies have already shown that the gene abnormality of phaeochromocytoma in MEN 2 is caused by abnormalities in the RET proto-oncogene on chromosome 10. The Von Hippel Lindau (VHL) gene is at chromosome 3p25.3. The neurofibromatosis gene is on chromosome 17. Mutations in p53 gene are also common in phaeochromocytomas especially in multiple and malignant cases but not benign solitary cases². The production of amines and other biologically active substances may be the final common pathway of a number of separate gene-driven

mechanisms. The genes are discussed in Chapter 6 and 16. These tumours are likely to be reclassified in the future on the basis of specific oncogenes or oncoproteins.

The German study in 1993 which reported that 19 of 82 (23%) unselected patients with phaeochromocytomas were carriers of familial disorders – 19% had VHL and 4% had MEN-2³ was challenging. These were unsuspected findings and the diagnoses were made on clinical, biochemical (pentagastrin tests, parathyroid hormone assays), CT or MRI scans of the brain, fundoscopy, CT of abdomen and ultra sound of the testes. Family members with known VHL or MEN 2 were screened with plasma and urine catecholamines and chromogranin, abdominal ultrasound, MRI and CT scans and MIBG scintigraphy. This was before the advent of gene testing. The authors concluded that all patients with phaeochromocytoma should be screened for MEN-2 and VHL disease to avoid further morbidity and mortality in family members. In the list of sporadic cases in this thesis, we have been unable to confirm these findings. However, many families in Ireland have proven reluctant to be screened when contacted and offered a cost-free study despite a careful explanation of the potential benefits.

As a policy standard, the gene abnormality in each phaeochromocytoma should be quantified and family studies carried out to find clinically silent gene mutation carriers. Seven patients collated in this thesis have had DNA gene studies up to January 2000. In the five years from January 1995, the National Centre for Medical Genetics at Our Lady's Hospital for Sick Children in Dublin received a total of 71 samples for MEN 2 testing. The laboratory does not possess the clinical information as to which of these patients had a phaeochromocytoma as undoubtedly some had. (Personal Communication dated 22 December 1999 from Professor Andrew Green, Professor of Medical Genetics at University College Dublin). A mutation in the RET protooncogene remains consistent within a family. Therefore family members at risk of disease need to be screened only once. This contrasts with the annual biochemical screens needed in familial thyroid carcinomas and the regular screens for phaeochromocytoma and hyperparathyroidism. Family members with the mutation can have appropriate treatment and those without a mutation and their descendants require no further follow-up action.

The National Cancer Registry Ireland should no longer consider phaeochromocytoma as a benign entity and should collate national incidence figures. If possible the registry should subdivide endocrine cancers. This would facilitate family gene studies to find people who are likely to be affected by MEN or VHL.

Recently published data on phaeochromocytoma screening in families at risk for MEN-2 and Von Hippel Lindau (VHL) disease reported on data from 26 patients with VHL disease and 8 with MEN-2a and 1 with MEN-2b who had histologically proven phaeochromocytoma and 47 patients with VHL and 3 with MEN-2 who had no radiological evidence of phaeochromocytoma. The patients were identified by family history and the diagnosis confirmed by identification of the germ-line mutation in the VHL tumour-suppressor gene or in the RET proto-oncogene in MEN-2. The data indicate that plasma normetanephrine and metanephrine is the best test at a sensitivity of 97% over urinary noradrenaline and adrenaline with a sensitivity of 74%. Interestingly urinary noradrenaline and adrenaline gave better sensitivities than the urinary metanephrines at 74% versus 65% respectively with corresponding specificities of 96% and 95%⁴. This clinical situation is a special case and the data differ from that found in studies of common sporadic phaeochromocytomas. The reference ranges used were derived from 125 normal subjects and 53 patients with hypertension for the plasma tests. 95% confidence intervals were established for plasma

levels of catecholamines, normetanephrine and metanephrine from these people. However the reference values for the urine tests were established in a different laboratory and clinical information of the subjects tested was not specified. The upper reference 95% limit for urinary noradrenaline was 80 µg/24 hr which is considerably less than our value of 152 µg/24 hr (900 nmol). If used routinely, this would create a huge false positive burden of continuing investigation in practice here. The upper 95% confidence limit for urinary adrenaline was 20 µg/24 hours, again much less than our value of 42.1µg/24 (230 nmol) hour with similar potential consequences.

Hypertension in pregnancy and phaeochromocytoma

Two patients in the thesis series of sporadic phaeochromocytomas were found in pregnancy. It is important to remain alert to the possibility of unusual causes of raised blood pressure in pregnancy as phaeochromocytoma may appear to be pre-eclampsia. One patient had classical symptoms but the other had a miscarriage, hypertension and placental insufficiency in a further pregnancy requiring early operative delivery which may be especially dangerous where there is the presence of an unsuspected phaeochromocytoma. Both patients had their tumours successfully removed after delivery. It is important to encourage and maintain good laboratory co-operation with the obstetric service to ensure that patients are appropriately investigated. Alpha blockers usually allow the baby to go to term. However there is an enhanced risk to both mother and foetus. In a review of 41 cases reported in the literature between 1988 and 1997, foetal loss was 11% and maternal mortality was 4%. The diagnosis was made antepartum in 83%. Before 24 weeks gestation, both medical therapy and surgical resection gave a good foetal outcome whereas, in later pregnancy, elective caesarian section followed by tumour surgery is the best option⁵. An earlier review of 42 cases in pregnancy between 1980 and 1987 found as maternal and foetal mortality of 17% and 26% respectively. However where the diagnosis was made antenatally, the maternal mortality was zero and foetal mortality was 15%⁶. A potentially fatal hypertensive attack may be induced by anaesthesia, labour and vigorous foetal movements. Alpha methyl dopa is most often used to treat pregnancy hypertension as short term treatment in the third trimester does not affect uteroplacental or foetal haemodynamics. Atenolol has adverse effects on placental function and impairs foetal growth⁷.

Pharmacological tests in phaeochromocytoma

Clonidine suppression tests have proved reliable in differentiating neurogenic hypertension from that due to phaeochromocytoma. False positive and negative tests do occur⁸. However, marked hypotension may be precipitated in some patients on antihypertensive drugs especially beta-adrenergic blockers. Provocative test should not be performed when the blood pressure is greater than 160/100 mm Hg. Glucagon, metaclopramide or histamine provocative tests are potentially dangerous and are in my view obsolete. The availability of biochemical tests for catecholamines and their metabolites has made the use of suppression tests using α-adrenergic blocking drugs (phentolamine) to screen patients also redundant. Despite this, clinical practice still reports their use in a minority of patients. In a large Italian study of 284 patients with phaeochromocytoma diagnosed between 1978 and 1997, 38 (13.4%) patients had clonidine suppression tests without adverse effects but there were two (5.3%) false negatives. Of the 21 glucagon provocation tests performed, acute hypertension caused their abandonment in 52.4%⁹.

While glucagon stimulation and clonidine suppression tests can enhance the accuracy of plasma catecholamine tests in phaeochromocytoma diagnosis, both have false positive and negative results¹⁰⁻¹².

Suppression tests with pentolinium have been used here recently as described in chapter 9.

Consent

Informed consent is necessary for every medical intervention. This is normally given by the patient. But when the patient is incapable of a rational decision, then permission is given or refused by the parent, guardian or in some circumstances by a close friend. If the patient subsequently refuses an intervention, then the patient's wishes are paramount.

Informed consent to investigation may not always be available. One patient in these studies from a psychiatric hospital who had a raised urine noradrenaline 1348 nmol/24 hr would not give consent to a CT scan following a normal abdominal ultrasound examination. The patient had malignant neuroleptic syndrome and returned to a psychiatric hospital. His wishes were respected.

While collecting samples from young children to establish a reference range for meaningful neuroblastoma screening, one mother refused to collect a sample from her 1 year old child as she was suspicious of all research despite careful explanation. The minimal risk attached to urine collections by a plastic stick on bag in young children is well within ethical guidelines adopted for children by the US Department of Health and Human Services in 1983¹³ from the recommendations of the National Commission for the Protection of Human Subjects in 1977¹⁴.

Consent is also an issue not raised in papers reporting compliance rates during neuroblastoma screening programs in children. Compliance in the Quebec screening study of a cohort of 476,603 infants from 1989 to 1994 was 91% and 74% for screens at three weeks and 6 months of age respectively¹⁵. The issue of compliance was specifically addressed in Quebec and special efforts were made to educate parents, health professionals and the public about the screening program¹⁶. In the Sapporo City screening study in Japan, compliance was 65.8% for 14 month old children in the period 1990 – 1994¹⁷. By contrast, a neuroblastoma screening programme in Texas between 1991 and 1994 had a very poor compliance rate of 4.8% overall. Children aged 5 – 10 months were offered screening using urine in filter paper. Compliance varied from a high of 53% at Houston's Women, Infants and Children clinic to a low of 4.7% by mail¹⁸. Why parents responded so differently is uncertain but special efforts to explain and educate are clearly essential in any medical intervention.

Creatinine

Serum creatinine is directly related to the total muscle mass. In conditions where the muscle mass is pathologically low, the urine creatinine may also be relatively low and lead to false positive "stat" urine catecholamine levels. This was shown in an 18 year old patient with arthrogryposis multiplex and hypertension who had amputations of both lower limbs. Using spot samples, his urine noradrenaline was 0.11, adrenaline 0.03 dopamine 2.26 and 1.16 all in nmol/mol creatinine and HMMA was 7.8, HVA 8.9 in $\mu\text{mol/mol}$ creatinine- all raised. The urine creatinines were 4818 and 5920 μmol which is below the adult range of 130 – 220 $\mu\text{mol/kg/day}$ (Normal reference ranges. N Eng J Med 1998;339:1063-72) usually 9,090 to

22,700 $\mu\text{mol}/24\text{ h}$. A mass was suspected on CT over his left adrenal but ^{126}I MIBG and octreotide scans were negative. A 24 hour collection showed urine noradrenaline 271, adrenaline 121, dopamine 2432 ($\text{mmol}/24\text{ hr}$) HMMA 17.6 and HVA 19.8 ($\mu\text{mol}/24\text{hr}$). It was concluded that the patient did not have a phaeochromocytoma and that the “spot” urines gave false positive values. The lower limb amputations lowered his muscle mass and body composition and thus creatinine production per Kg body weight.

Reference ranges of urine catecholamines and their metabolites which are related to the urine creatinine should ideally be age-related as muscle mass in adults decreases with age and varies with sex and diet.

Potential dangers

Clinical respect for the potential hazards of catecholamine excess should be stressed.

Noradrenaline causes cardiac muscle hypertrophy through stimulation of α_1 and β adrenergic receptors and by activation of the renin-angiotensin-aldosterone system. Noradrenaline is directly toxic to myocardial cells. This is mediated through the induction of calcium overload and apoptosis¹⁹. This apoptotic effect can be prevented by non selective β blockade or by combined α and β blockade. Reversible cardiomyopathy due to catecholamines may occur²⁰ and end-stage cardiomyopathy requiring heart transplantation has been reported due to an occult phaeochromocytoma²¹. Lethal lactic acidemia may also supervene as detailed in Chapter 6. Orthostatic hypotension may be a hazard especially in older patients. Systolic blood pressure decreases more on standing in patients with phaeochromocytoma than in normotensive or essential hypertensive controls. Altered sympathetic vascular regulation is the cause and tumour excision normalises the autonomic physiology²².

Imaging investigations.

As these investigations are expensive, they should be carried out selectively. The clinical history should be suggestive and/or the urine biochemical screen should be positive or equivocal. Imaging by ultrasound is painless, convenient and widely available in district general hospitals. CT scans are usually available and expert interpretation from a remote site is available by electronic data transmission to hospitals which act as referral centres for endocrine tumours. As about 90% of phaeochromocytomas are intra-adrenal, CT alone is often sufficient for diagnosis. MRI is also useful and data can similarly be transferred for interpretation. Labelled MIBG gives a diagnostic sensitivity in the high 80%^s. The ^{123}I label gives better image quality than ^{131}I . It is important that a drug history is taken and that drugs which may cause false negative scans such as labetalol, verapamil, tricyclic antidepressants, phenothiazines, butyrophenones and adrenergic neuron blockers are not in the patients system (see Chapter 12). The requirement for patient isolation following the use of ^{123}I MIBG or ^{131}I MIBG scintigraphy means that single rooms must be used for four days after injection of the radiopharmaceutical. This is necessary to protect all others from inadvertent irradiation. 70 – 90% of ^{131}I MIBG appears in the urine within four days. There is a place for each imaging modality in clinical practice. Their association gives the best results. MIBG is especially useful to search for metastases and for second primary tumours.

Suggested protocol for the diagnosis of a possible phaeochromocytoma.

- (a) A single 24 hour urine collection for urinary free catecholamines or total metanephrines should be done when the clinical picture suggests low risk. The reference values should be derived from a patient population with essential hypertension.
- (b) Three collections over three consecutive days where the risk is higher ie where there are paroxysms of hypertension, headache, sweating, palpitations or other suggestive symptoms.
- (c) If plasma normetanephrine and metanephrine estimation is available, a plasma sample for these analytes should be taken in the morning from an indwelling catheter in an antecubital vein after resting supine for 20 minutes. No smoking or methylxanthine containing food products should be taken after the previous midnight. Any drug therapy should be noted to aid interpretation.
- (d) Short or spot urines can be used for children, or for ill or uncooperative patients.
- (e) A random 1-hour morning urine for normetanephrines +/- catecholamines may be done in a day-case ward for cost-efficiency.
- (f) Plasma catecholamines may be used when urines remain normal but clinical suspicion is high. They may also be used in patients with renal failure.
- (g) Abdominal ultrasound and CT and or MRI scanning where the biochemical screen is positive or if clinical suspicion remains high despite a normal urine result.
- (h) MIBG scan if CT is positive but urinary values are equivocal or if CT is normal despite high urine biochemical screening values.
- (i) MIBG pre-operative where a phaeochromocytoma has been diagnosed and there is a clinical suspicion of a metastasis.
- (j) Venous sampling for plasma catecholamines and metanephrines may be required when imaging techniques are equivocal.
- (k) Positron emission tomography (PET) using FDG (Chapter 12) may be used especially as an adjunct in the search for metastases.
- (l) The clonidine suppression test may have a role in separating neurogenic hypertension from phaeochromocytoma when the urinary catecholamines are slightly increased and the imaging modalities are negative.
- (m) A reference range derived from normotensive subjects should be used where phaeochromocytoma is being sought in a family where MEN-2 or Von Hippel Lindau disease is proven.

This protocol differs in some details from that published in 1995²³.

Follow-up

Following surgical excision of phaeochromocytoma, there should be annual reviews of blood pressure and symptoms together with the measurement of urinary catecholamines to check for recurrence, metastases or the development of new tumours. Follow-up at six monthly intervals for 15 years or perhaps for life following surgery for 'benign' phaeochromocytoma has been suggested²⁴ but patient compliance with such a regime is unlikely. Imaging should be used where suspicions are aroused. At diagnosis, all patients should be formally tested to out-rule MEN, von Hippel Lindau disease and neurofibromatosis, type 1. The patients should be evaluated for genetic mutations and families should be evaluated to ensure that familial syndromes are discovered. DNA ploidy does not appear to have a role in prognosis in the individual case. The role of chromogranins as a follow-up tumour marker also needs evaluation.

In screening patients at risk for pheochromocytoma due to the presence of MEN-2, or Von Hippel Lindau disease in a relative, a reference range using only normotensive subjects should be used. This differs from screening pheochromocytoma patients from the general mass of hypertensive individuals where hypertensives have been used to reduce the burden of a high number of false positives which would occur should a normotensive healthy population be used to calculate the reference interval. The reference values used by Neumann et al found tumours in 36 of 79 subjects at high risk of pheochromocytomas. Their reference limits were considerably lower than those used in this thesis²⁵ (see chapter 8). Eisenhofer et al recently showed that plasma normetanephrine and metanephrine are useful in screening for pheochromocytoma in patients with genetically proven MEN-2 or Von Hippel Lindau. Their sensitivity for the detection of tumours in this context was 97%²⁶.

Malignancy

The presence of a chromaffin cell invasion or dissemination at anatomical sites where chromaffin tissue is normally not present is the only sure definition of malignancy in pheochromocytoma. There are no well-defined reliable histopathological criteria to diagnose malignancy in this condition. Nuclear pleomorphism, mitotic figures, vascular invasion, cortical extension, necrosis and immunocytochemical characteristics have not proven useful in determining malignancy. Around 13 % are reported to be malignant but various series have quoted rates from 5 – 60%²⁷.

There are many conflicting claims in the literature. The most recent report is from a long-term follow-up over a 35 year period of a series of 86 patients with 85 benign and 10 malignant pheochromocytomas²⁸. Comparisons were made between the data from the benign and the malignant tumours. HMMA tended to be higher in malignant tumours but there were no differences in the urinary noradrenaline or adrenaline. Malignant pheochromocytomas were more often extra-adrenal than adrenal. Tumours weighting more than 80g were more often malignant ($p = 0.0001$) Dopamine tumour concentration was higher in malignant tumours ($p = 0.01$). Pre-operative urinary dopamine >5250 nmol/24 hr correlated with malignancy ($p < 0.0001$) Persistent post-operative hypertension also increased the likelihood of malignancy ($p = 0.001$). The authors conclude that patients with these characteristics should have more frequent follow-ups to identify metastatic spread early should this occur. By contrast Goldstein et al reported the same risk of malignancy in adrenal and extra-adrenal pheochromocytomas in 108 pheochromocytomas in 104 patients during the period 1950 to 1998²⁹. The contradictory literature on dopamine and HVA in relation to malignancy is discussed in chapter 10.

With regard to DNA ploidy, tetraploid and aneuploid tumours are associated with malignancy but diploidy does not preclude malignancy (chapters 10 and 11). Thus ploidy does not really assist in the treatment of the individual patient, assuming that a policy of systematic follow-up of all patients is in place.

Recurrence of pheochromocytoma may occur from incomplete removal of the tumour at surgery, the development of a second primary in the other adrenal gland, or at another site, and from intraoperative disruption of the tumour with implantation or embolisation of tumour cells. Recurrence following the removal of a seemingly benign pheochromocytoma may take 6 months to 21 years or longer³⁰. In one series the average time from primary surgery to recurrence was 8 years³¹. As well as chemotherapy and irradiation with labelled MIBG for the

treatment of metastatic pheochromocytoma, alpha methyl tyrosine may be used to block the synthesis of catecholamines and relieve symptoms³². In this thesis series, there were two cases of malignant pheochromocytoma and one malignant paraganglioma. This proportion (9.1%) is as expected. Longer follow-up may reveal other tumour recurrences in the future.

Expert system reporting in pheochromocytoma

Data interpretation by expert system has been developed by the Australian tertiary referral centre for pheochromocytoma diagnosis from a data base of 75 patients with pheochromocytoma and >8000 'nontumour' patients. The system interprets individual patient data in relation to reference intervals derived from the whole 'nontumour' population that is held in the database. Their REPCAT application examines 11 analytes and specific data from the sample request for biochemical investigations of pheochromocytoma and produces a report that is equivalent to that from an expert pathologist³³. The analytes that are currently used in the Australian system are noradrenaline, adrenaline, dopamine, their *o*-methyl analogs (if patient is taking aldomet), 3,4-dihydroxyphenyl glycol (DHPG), 3,4-dihydroxyphenylacetic acid (DOPAC), normetanephrine, metanephrine and creatinine. It has been 100% sensitive in the diagnosis of pheochromocytoma after 5 years of laboratory use. It is likely that such systems will be available to all investigating clinicians in the future which will aid diagnostic efficiency.

Carcinoid tumours

In studying those 24 cases detailed in chapter 14, "no firm conclusion can be drawn in many of these cases as to whether the increased 5-HIAA is a coincidence or an indicator of disease." Radiolabelled MIBG, octreotide and VIP scintiscans are relatively under-utilized. However the carcinoid syndrome is rare in clinical practice and other biochemical markers may be more appropriate in the investigation of flushing after a careful drug history has been taken. Common drugs such as paracetamol (acetaminophen), atenolol, indomethacin, naproxen, diazepam and glyceryl guaicolate (found in some cough mixtures) may cause a false positive raised urinary 5-HIAA level as may bananas, walnuts, coeliac disease, Whipples disease and gut bacterial overgrowth syndromes. Flushing is a relatively common feature of pheochromocytoma but 5-HIAA is usually within the reference limits.

Neuroblastoma

Neuroblastoma is the most common extracranial solid tumour of childhood. It has a longterm survival of only 15%. Recent advances have been made in the treatment of high risk neuroblastoma which form the basis of active treatment in these ill patients. Patients received initial therapy with cisplatin, doxorubicin, etoposide and cyclophosphamide for five cycles, plus surgery and radiotherapy for gross residual disease. Those without disease progression who had myeloablative chemotherapy, total-body irradiation, and transplantation of autologous bone marrow purged of neuroblastoma cells had a survival advantage over those who received a further three courses of chemotherapy alone; 3 year event free survival was 34+/- 4% versus 22+/-4% ($p=0.34$). Those patients who were without disease progression after transplantation or extra chemotherapy were randomised to given either no further therapy or 13-*cis*-retinoic acid. The 3 year event free survival was 46+/-6% versus 29+/-5% ($p=0.027$) in favour of the active regime³⁴. Only 8% of patients survived more than 3 years after relapse. A serum ferritin of 143 ng/ml was used as a boundary between higher and lower risk patients. This was the only prognostic biochemical marker reported in the study.

The effectiveness and appropriateness of screening for neuroblastoma is not free of controversy. The Osaka group in Japan³⁵ evaluated trends in incidence and survival in neuroblastoma before and after the introduction of systematic screening during the period 1970 to 1984. The incidence at birth per 100,000 live births was 2.30 in 1970-89 before screening rising to 19.89 per 100,000 live births in 1988-89 following the introduction of screening. The incidence rate in children at 1 and 2-4 years also rose with screening. The mortality rate in children aged 1 – 4 years per 100,000 live births slightly decreased from 3.87 in 1970-79 to 3.30 in 1988 – 89. This was most likely due to improvements in the treatment regimes. The authors felt strongly that mass screening for neuroblastoma causes harm due to overdiagnosis and has little effect on decreasing the incidence and mortality of neuroblastoma at 1 – 4 years of age²⁹. Data from the mass screening programme in Sapporo City, Japan found that mass screening of children at 14 months detects cases that were missed at 6 months and found that the detection rate was higher than expected from the natural incidence¹⁷. Another Japanese group in Iwatsuki reported on the spontaneous regression in neuroblastoma and concluded that this was a relatively common phenomenon. They suggested a wait-and-see strategy with careful observation by abdominal ultrasonography and monthly urinary HMMA and HVA for selected stage 1 and 11 tumours found in children screened at 6 months of age³⁶. The question of stopping the Japanese screening program has clearly surfaced. In a review of the Quebec screening project referred to above, the authors concluded that the majority of the unfavourable histology neuroblastomas that developed were included in the group of screening negative cases and could not be detected by screening at the age of 3 weeks and/or at 6 months³⁷.

From these data and the discussion on the clinical investigation in neuroblastoma in Chapter 18, there is no compelling case to introduce neuroblastoma screening in children at about 6 months of age in Europe and in the British Isles. Thus the 1991 conclusion of the consensus statement from the American Cancer Society on neuroblastoma screening that “Physicians and the public need to know that the effectiveness of mass screening remains unproven³⁸” continues to be the position.

Future direction

In neuroblastoma, the use of the metabolites, metanephrine and normetanephrine as a diagnostic tool and their possible use as tumour markers in monitoring the effects of chemotherapy will be studied. The chromatogram in Figure 7 of chapter 4 (Asted section) is a representation of the rationale. Biological and analytical interferences by drugs will be further studied.

Method changes/costs

The comment by Wolthers et al that ELISA kits such as that reported for urinary metanephrine and normetanephrines by his group is likely to replace chromatographic methods for screening for phaeochromocytoma may be correct³⁹. Time, assay performance and cost will tell. A recent study has shown that therapeutic drugs have not interfered in the immunoassays of urinary free catecholamines and total metanephrine⁴⁰. The authors assert that the combination of these assays is suitable as an initial screen for phaeochromocytoma as the methods are more accessible to clinical laboratories than HPLC. For neuroblastoma, simpler methods such as enzyme immunoassay or chemiluminescence⁴¹ may make screening technically easier.

At January 2000, costings for enzymeimmunoassay ELISA kits from Immunodiagnostics of Boldon, Tyne and Wear, England are noradrenaline, adrenaline, normetanephrine and metanephrine £295 for 96 wells in each case. This will give 88 samples with 6 standards and 4 controls. Therefore unit cost is £3.35 per analyte.

For radioimmunoassays from the same company, for adrenaline and noradrenaline the cost is £210 for 50 tubes, for dopamine it is £180 for 50 tubes and for combined total metanephrines it is £325 per 100 tubes. With 8 tubes for standards (4 x 2 ie. duplicates) and 4 x 2) controls that leaves 34 tubes for patient specimens, which when done in duplicate which is standard practice, this gives 17 patient specimens per £210 or £12.35 per test. For noradrenaline, adrenaline £24.70 per patient plus £7.70 for dopamine giving reagent costs of £32.40 for the package of noradrenaline, adrenaline and dopamine.

HPLC method costs are IR£400 per column. These last for about 800 patients each and we have been using two per year. The cost of the mobile phase is minimal as it is made up in-house. Each sample has about 50p in consumables cost and this gives either noradrenaline, adrenaline and dopamine or HMMA, HVA and 5-HIAA. HPLC and electrochemical detector capital cost is about £60,000 or about £6,000 per year for ten years or £3.75 per sample. The electrochemical detector requires replacement of both the conditioning and analytical cells yearly at a cost of approximately £2,400 or £1.50 per sample. Thus the approximate capital cost is £5.25 per test totaling £5.75 per test package. This is cheaper than the alternatives but the cost of two senior technicians must be factored into the HPLC costs as this would be much less expensive using microtitre wells as the analysts' time factor is much shortened..

The ASTED HPLC system including the electrochemical detector costs £26,000 which is £2,600 written off over ten years or £1.62 per test. The system cells may last longer (Personal communication Dr J Dutton, Liverpool , analytical columns lasting three times longer). Given the same electrochemical detector costs of £1.50 per sample and the same column costs of £0.50 per sample, the ASTED package is £3.62 per sample which will also include metanephrines. There is also much less analyst time involved when the system has been set up.

I conclude that should the costs of ELISA kits fall to approximately those of HPLC analysis, these kits are likely to supercede HPLC methods in the future. However, urine dopamine should be part of the package as rare dopamine secreting phaeochromocytomas continue to be reported in the literature^{42,43}. Dopamine is however essential for neuroblastoma screening. The laboratory policy of avoiding radio-isotopic methods will continue.

Will the potential of plasma metanephrines in phaeochromocytoma be vindicated?

A 1995 combined report from the National Institutes of Health, Maryland, US, Goteborg, Sweden and Nijmegen in Holland noted that the measurement of plasma metanephrine and normetanephrine gave 100% sensitivity for pheochromocytoma diagnosis and a negative predictive value of 100% in 52 patients with proven phaeochromocytomas⁴⁴ should be confirmed as most patients would prefer the convenience of a single blood test over a 24 hour urine collection. It should be noted that if hypertensive controls only had been used by this group to calculate their reference range, then the above sensitivity would have been 98%. Using receiver operating characteristic (ROC) curves, the sensitivity of tests using metanephrines remained higher than that of tests using catecholamines irrespective of

changes in the upper reference limit used. A restatement of the advantages of using plasma normetanephrine and metanephrine over plasma catecholamines was made by one of the authors in a 1998 paper in Swedish⁴⁵. If this work is reaffirmed, normal plasma metanephrines would serve as a excluder of the diagnosis of phaeochromocytoma in contrast to the plasma catecholamines which had a much higher rate of false negative results. Lenders et al also noted that in phaeochromocytoma, the metanephrines had higher values than catecholamines with reference to the upper reference limit value.

In patients with phaeochromocytoma, 93.9% of the elevated levels of plasma free normetanephrines and 97.7% of the plasma level of metanephrine were derived from catechol-O-methyltransferase activity in tumour cells converting catecholamines to metanephrines before entry into the circulation. The high levels were not from metabolism of catecholamines after reaching the plasma. Levels of free metanephrines in phaeochromocytomas were 10,000 times higher than plasma concentrations in the same patients before surgical removal of the tumour⁴⁶. Some tumours do not secrete catecholamines but all appeared to metabolize catecholamines to free metanephrines. This may explain the reported high sensitivity of plasma free metanephrines over other tests in the diagnosis of phaeochromocytoma. Eisenhofer et al also stated that plasma free metanephrines may provide an indication of tumour size unlike the plasma catecholamines. Mornex et al also reported that plasma total metanephrines were significantly correlated with tumour size ($r = 0.83; p < 0.001$), however the study included only 12 data points⁴⁷. This thesis found no relationship between tumour size and urine catecholamines. In a study of 16 patients with phaeochromocytoma in which three dimensional data on the excised phaeochromocytomas were detailed, no correlation could be demonstrated between the size of the tumour and the plasma levels of noradrenaline or adrenaline or between tumour size and the 24 hour urinary excretion of noradrenaline, adrenaline, normetanephrine or metanephrine using Pearson's correlation coefficient⁴⁸. However false positive total plasma metanephrine results may occur in patients with chronic renal disease where there was little discrimination between phaeochromocytoma patients and those on haemodialysis⁴⁹. In the future, the use of plasma metanephrines will be formally studied using the ASTED HPLC method.

With the pressure for increasingly efficient use of resources, the 1-hour urine collection for normetanephrine, metanephrine, noradrenaline and adrenaline reported in nmol/hour may be an equally valid method for disease screening as the reported sensitivity of the metanephrines is 100%⁵⁰ (see chapter 8). Clearly this work should be confirmed as it would allow an early answer especially if an enzymeimmunoassay method was used.

Mutation analysis

Fast nonradioactive methods for mutation analysis in exons 10,11 and 16 of the RET protooncogene have been developed⁵¹. At present, DNA sequence analysis is done to confirm the mutation. These and similar developments will aid the spread of detailed precise diagnostic methods to routine laboratories.

References

1. Sadeg N, Francois G, Petit B, Dutertre-Catella H, Dumontet M. Automated liquid-chromatographic analyzer used for toxicology screening in a general hospital: 12 months' experience. *Clin Chem* 1997;43:498-504.
2. Yoshimoto T, Naruse M, Zeng Z, Nishikawa T, Kasajima T, Toma H, Yamamori S, Matsumoto H, Tanabe A, Naruse K, Demura H. The relatively high frequency of p53 gene mutations in multiple and malignant pheochromocytomas. *J Endocrinol* 1998;159:247-55.
3. Neumann HPH, Berger DP, Sigmund G, Blum U, Schmidt D, Parmer RJ, Volk B, Kirste G. Pheochromocytomas, multiple endocrine neoplasia type 2, and von Hippel-Lindau disease. *N Engl J Med* 1993;329:1513-8.
4. Eisenhofer G, Lenders JWM, Linehan WM, Walther MM, Goldstein DS, Keiser HR. Plasma normetanephrine and metanephrine for detecting pheochromocytoma in von Hippel-Lindau disease and multiple endocrine neoplasia type 2. *N Engl J Med* 1999;340:1872-9.
5. Ahlawat SK, Jain S, Kumari S, Varma S, Sharma BK. Pheochromocytoma associated with pregnancy: case report and review of the literature. *Obstet Gynecol Surv* 1999;54:728-37.
6. Harper MA, Murnaghan GA, Kennedy L, Hadden DR, Atkinson AB. Pheochromocytoma in pregnancy. Five cases and a review of the literature. *Br J Obstet Gynaecol* 1989;96:594-606.
7. Wood AJJ. Treatment of hypertension in pregnant women. *N Engl J Med* 1996;335:257-65.
8. Taylor HC, Mayes D, Anton AH. Clonidine suppression test for pheochromocytoma: Examples of misleading results. *J Clin Endocrinol Metab* 1986;63:238-42.
9. Mannelli M, Ianni L, Cilotti A, Conti A. Pheochromocytoma in Italy: a multicentric retrospective study. *Eur J Endocrinol* 1999;141:619-24.
10. Bravo EL, Tarazi RC, Fouad FM, Vidt DG, Gifford RW jr. Clonidine-suppression test; a useful aid in the diagnosis of pheochromocytoma. *N Engl J Med* 1981;305:623-6.
11. Grossman E, Goldstein DS, Hoffman A, Keiser HR. Glucagon and clonidine testing in the diagnosis of pheochromocytoma. *Hypertension* 1991;17:733-41.
12. Stein PP, Black HR. A simplified diagnostic approach to pheochromocytoma. A review of the literature and report on one institution's experience. *Medicine* 1991;70:46-66.

13. 48 Fed Reg 9818, March 8, 1983, codified at 45 CFR # 46.406-46.408,1998.
14. National Commission for the Protection of Human Subjects of Biomedical and Behavioural research. Report and recommendations: research involving children. Washington DC: Government printing Office, 1977.
15. Woods WG, Tuchman M, Robison LL, Bernstein M, Leclerc J-M, Brisson LC, Brossard J, Hill G, Shuster J, Luepker R, Byrne T, Weitzman S, Bunin G, Lemieux B. A population based study of the usefulness of screening for neuroblastoma. *Lancet* 1996;348:869-70.
16. Campion P, Woods WG, Lemieux B. Compliance in a screening program for neuroblastoma. *Prev Med* 1998;27:590-6.
17. Nishi M, Miyake H, Takeda T, Hanai J, Kikuchi Y, Hiramata T. Mass screening for neuroblastoma targeting children age 14 months in Sapporo City: a preliminary report. *Cancer* 1998;82:1973-7.
18. Ater JL, Gardner KL, Foxhall LE, Therrell BL jr, Bleyer WA. Neuroblastoma screening in the United States: results of the Texas Outreach Program for neuroblastoma screening. *Cancer* 1998;82:1593-1602.
19. Mann DL, Kent RL, Parsons B, Cooper G iv. Adrenergic effects on the biology of the adult mammalian cardiocyte. *Circulation* 1992;85:790-4.
20. Gatzoulis KA, Tolis G, Theopistou A, Gialafos JH, Toutouzas PK. Cardiomyopathy due to pheochromocytoma. A reversible entity. *Acta Cardiol* 1998;53:227-9.
21. Wilkenfeld C, Cohen M, Lansman SL, Courtney M, Dische MR, Pertsemlidis D et al . Heart transplantation for end-stage cardiomyopathy caused by an occult pheochromocytoma. *J Heart Lung Transpl* 1992;11:363-6.
22. Munakata M, Aihara A, Imai Y, Noshiro T, Ito S, Yoshinaga K. Altered sympathetic and vagal modulations of the cardiovascular system in patients with pheochromocytoma: their relations to orthostatic hypotension. *Am J Hypertension* 1999;12:572-80.
23. Tormey WP, Fitzgerald. Lack of uniformity in the clinical approach to the interpretation of urinary catecholamines and their metabolites. *Ir J Med Sci* 1995;164:146-50.
24. Sparagana M. Late recurrence of benign pheochromocytomas: the necessity for long term follow-up. *J Surg Oncol* 1988;37:140-6.
25. Neumann HPH, Berger DP, Sigmund G, Blum U, Schmidt D, Parmer RJ, Volk B, Kirste G. Pheochromocytomas, multiple endocrine neoplasia type 2, and von Hippel-Lindau disease. *N Engl J Med* 1993;329:1531-8.
26. Eisenhofer G, Lenders JWM, Linehan WM, Walther MM, Goldstein DS, Keiser HR. Plasma normetanephrine and metanephrine for detecting pheochromocytoma in von Hippel-Lindau disease, and multiple endocrine neoplasia type 2. *N Engl J Med* 1999;340:1872-9.

27. Manger WM, Gifford RW. Management of malignant pheochromocytoma. In Clinical and experimental pheochromocytoma. 2nd ed. Blackwell Scientific, Cambridge, Mass, USA. Chapter 8, 433-46.
28. John H, Ziegler WH, Hauri D, Jaeger P. Pheochromocytomas: can malignant potential be predicted? *Urology* 1999;53:679-83.
29. Goldstein RE, O'Neill JA jr, Holcomb GW 3rd, Neblett WW 3rd, Oates JA, Brown N, Nadeau J, Smith B, Page DL, Abumrad NN, Scott HW jr. Clinical experience over 48 years with pheochromocytoma. *Ann Surg* 1999;229:755-64.
30. Traub YM, Rosenfeld JB. Malignant pheochromocytoma with pleural metastases of unusually long duration. *Chest* 1970;58:546-50.
31. Mahoney EM, Harrison JH. Malignant pheochromocytoma: clinical course and treatment. *J Urol* 1977;118:225-9.
32. Engelman K, Horwitz D, Jequier E, Udenfriend S, Sjoersdsma A. Biochemical and pharmacologic effects of alpha-methyl tyrosine in man. *J Clin Invest* 1968;47:568-76.
33. Smythe GA, Drew C. REPCAT: desktop expert system for interpreting and validating laboratory data for pheochromocytoma diagnosis with the database application Omnisttm. *Clin Chem* 1997;43:134-40.
34. Matthay KK, Villablanca JG, Seeger RC, Stram DO, Harris RE, Ramsay NK, Swift P, Shimada H, Black CT, Brodeur GM, Gerbing RB, Reynolds CP. For the Children's Cancer Group. Treatment of high risk neuroblastoma with intensive chemotherapy, radiotherapy, autologous bone marrow transplantation and 13-cis-retinoic acid. *N Engl J Med* 1999;341:1165-73.
35. Ajiki W, Tsukuma H, Oshima A, Kawa K. Effects of mass screening for neuroblastoma on incidence, mortality, and survival rates in Osaka, Japan. *Cancer Causes Control* 1989;9:631-6.
36. Yamamoto K, Hanada R, Kikuchi A, Ichikawa M, Aihara T, Oguma E, Moritani T, Shimanuki Y, Tanimura M, Hayashi Y. Spontaneous regression of localized neuroblastoma detected by mass screening. *J Clin Oncol* 1998;16:1265-9.
37. Kawakami T, Monobe Y, Monforte H, Woods WG, Tuchman M, Lemieux B, Brisson L, Bernstein M, Brossard J, Leclerc JM, Byrne TD, Triche TJ, Shimada H. Pathology review of screening negative neuroblastomas: a report from the Quebec Neuroblastoma Screening Project. *Cancer* 1998;83:575-81.
38. Murphy SB, Cohn SL, Craft AW, Woods WG, Sawada T, Castleberry RP, Levy HL, Prorok PC, Hammond GD. Do Children benefit from mass screening for neuroblastoma? Consensus statement from the American Cancer Society Workshop on Neuroblastoma Screening. *Lancet* 1991;337:344-5.

39. Wolthers BG, Kema IP, Volmer M, Wesemann R, Westermann J, Manz B. Evaluation of urinary metanephrine and normetanephrine enzyme immunoassay (ELISA) kits by comparison with isotope dilution mass spectrometry. *Clin Chem* 1997;43:114-20.
40. Wassall J, Reed P, Kane J, Weinkove C. Freedom from drug interference in new immunoassays for urinary catecholamines and metanephrines. *Clin Chem* 1999;45:2216-23.
41. Nozaki O, Kawamoto H, Moriyama H. Total free catecholamines assay by identification of its two functional groups and micro-flow injection chemiluminescence. *Luminescence* 1999;14:369-74.
42. Yasunari K, Kohno M, Yoshikawa J. A dopamine-secreting pheochromocytoma. *Am J Med* 1999;106:599-600.
43. Bartolacci M, Bartolacci S, Leombruni E, Annunziata A, Di Rienzo M, Picardi N. Adrenal incidentaloma: a dopamine-secreting pheochromocytoma with an atypical clinical picture. A case report and review of the literature. *Ann Ital Chir* 1998;69:805-14.
44. Lenders JW, Keiser HR, Goldstein DS, Willemsen JJ, Friberg P, Jacobs MC, Kloppelborg PW, Thien T, Eisenhofer G. Plasma metanephrines in the diagnosis of pheochromocytoma. *Ann Intern Med* 1995;123:101-9.
45. Friberg P. Plasma metanephrine measurements make the diagnosis of pheochromocytoma easier. *Lakartningen* 1998;95:2482-5. (paper in Swedish)
46. Eisenhofer G, Keiser H, Friberg P, Mezey E, Huynh TT, Hiremagalur B, Ellingson T, Duddempudi S, Eijsbouts A, Lenders JW. Plasma metanephrines are markers of pheochromocytoma produced by catechol-O-methyltransferase within tumors. *J Clin Endocrinol Metab* 1998;83:2175-85.
47. Mornex R, Peyrin L, Pagliari R, Cottet-Emard J-M. Measurement of plasma methoxyamines for the diagnosis of pheochromocytoma. *Horm Res* 1991;36:220-6.
48. Peaston RT, Lennard TWJ, Lai LC. Overnight excretion of urinary catecholamines and metabolites in the detection of pheochromocytoma. *J Clin Endocrinol Metab* 1996;81:1378-84.
49. Marini M, Fathi M, Valloton M. Determination of serum metanephrines in the diagnosis of pheochromocytoma. *Ann Endocrinol (Paris)* 1995;54:337-42.
50. Oishi S, Sasaki M, Ohno M, Sato T. Urinary normetanephrine and metanephrine measured by radioimmunoassay for the diagnosis of pheochromocytoma: utility of 24-hour and random 1-hour urine determinations. *J Clin Endocrinol Metab* 1988;67:614-8.
51. Siegelman M, Mohabeer A, Fahey TJ 111, Tomlinson G, Mayambala C, Jafari S, Noll WW, Thibodeau SN, Dawson DB. Rapid, nonradioactive screening for mutations in exons 10,11,and 16 of the RET protooncogene associated with inherited medullary thyroid carcinoma. *Clin Chem* 1997;43:453-7.

Publications associated with this thesis

Part of the subject matter of this thesis has been published as listed below

1. Fitzgibbon M, FitzGerald RJ, Tormey WP, O'Meara A, Kenny D. Reference values for urinary HMMA, HVA, noradrenaline, adrenaline and dopamine excretion in children using random urine samples and HPLC with electrochemical detection. *Ann Clin Biochem* 1992;29:400-4.
2. O'Neill G, Tormey W P, Farrell MA, Fitzgerald RJ, Finucane J, Phaeochromocytoma and lethal lactic acidemia. *Ir Med J* 1993;86:211.
3. Fitzgibbon MC, Tormey WP. Paediatric reference ranges for urinary catecholamines/metabolites and their relevance in neuroblastoma diagnosis. *Ann Clin Biochem* 1994;31:1-11.
4. O'Meara A, Tormey W, Fitzgerald R J, Fitzgibbon M, Kenny D, Interpretation of random urinary catecholamines and their metabolites in neuroblastoma. *Acta Paediatr* 1994;83:88-92.
5. Tormey WP, Fitzgerald RJ. Phaeochromocytoma-a laboratory experience. *Ir J Med Sci* 1995;164:142-5.
6. Tormey WP, FitzGerald RJ. Lack of uniformity in the clinical approach to the interpretation of urinary catecholamines and their metabolites. *Ir J Med Sci* 1995;164:146-50.
7. Tormey WP. Phaeochromocytoma diagnosis. *Ann Clin Biochem* 1995;32:595-7.
8. Tormey WP, Fitzgerald RJ. The clinical and laboratory correlates of an increased urinary 5-hydroxyindoleacetic acid. *Postgrad Med J* 1995;71:542-5.
9. Tormey WP. Repetition of data in publications on DNA flow cytometry in phaeochromocytoma. *Clin Endocrinol* 1998;49:692-3.
10. Tormey WP, Kay EW, Hickey DP. Renal cell carcinoma presenting as a pseudo-carcinoid tumour. *Eur J Gastroenterol Hepatol* 1999;11:1059-60.
11. Tormey WP, Carney M, Fitzgerald RJ. Catecholamines in urine after death. *Forensic Sci Int* 1999;103:67-71.
12. Tormey WP, Kealing F, Lee MJ. ¹²³I-metaiodobenzylguanidine scans in phaeochromocytoma diagnosis: current clinical practice and relationship to biochemical screening. *Int J Clin Pract* 1999;53:428-32.
13. Tormey WP, FitzGerald RJ, Thomas G, Kay EW, Leader MB. Catecholamine secretion and ploidy in phaeochromocytoma. *Int J Clin Pract* 2000;54: in press

Part of the subject matter of this thesis was presented at scientific meetings and an abstract is listed below.

1. Fitzgibbon M, Fitzgerald R, Tormey W. Differing responses between two electrochemical detectors in the simultaneous analysis of HMMA, 5-HIAA and HVA. Proc ACB National Meeting 1990;111.(abstract)