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Characterisation and analysis of β-aminoketones as potential prodrugs for amines

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B. Sc. (Chem. Eng., IST), M. Sc. (FFL)

A thesis presented to the University of Dublin for the degree of
Doctor of Philosophy in Pharmaceutical Chemistry

Based on research carried out under the supervision of
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Trinity College Dublin.

2004

DECLARATION

This thesis has not been presented as an exercise for a degree at any other university. The work described, except where duly acknowledged, was carried out by me entirely.

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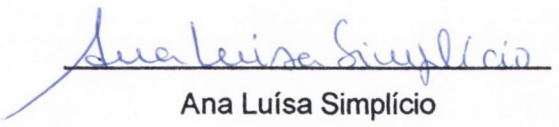

Ana Luísa Simplício

ABSTRACT

The aim of the work described in this thesis is to evaluate the potential of β-aminoketones as amine prodrugs. These compounds are usually stable in acidic conditions while they cleave into the parent amine and an α,β-unsaturated ketone at neutral to basic pH. The rates of liberation of the amine may be suitable for application of these compounds as prodrugs.

Chapter 1 describes the principal strategies normally used for the design of prodrugs. Published approaches to amine prodrugs are described with particular detail.

Chiral and achiral methods were developed for the analysis of certain β-aminoindanones and related compounds. This work, which is described in Chapter 2, lead to the discovery that some β-aminoindanones degrade, in physiological conditions, at rates consistent with their application as prodrugs for amines.

In Chapter 3 the kinetic tests performed on new β-aminoindanones are described. This confirms the applicability of the system to a wider range of primary and secondary amines.

Chapter 4 reports the synthesis and testing of new compounds derived from different carbonyl promoieties to evaluate any structure reactivity relationships and test the generality of the system.

In Chapter 5, some β-aminoketone systems are applied to produce prodrugs of dopamine and some aspects of their usefulness are evaluated.

Conclusion remarks and future work are presented in Chapter 6.

Finally, in Chapter 7, the synthesis and analytical methodologies used during the course of this work are described. The identification characteristics of the compounds synthesised are given.

ACKNOWLEDGMENTS

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To Dr. Neil Frankiesh and Dr. Helen Sheridan I am very obliged due to the donation of some test compounds. To Dr. Helen Sheridan and Dr. John Walsh I must also thank for guiding me through my first steps on the path of synthesis.

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To Mum and Dad, it is impossible to express the extent of my thankfulness, but to them I have to write in Portuguese. I apologise to those who can't read it:

Queridos paizinho e mãezinha, não tenho palavras para agradecer todo apoio e confiança que, como sempre, demonstraram. Eu sei que estes anos foram provavelmente mais difíceis para vocês do que para mim. Obrigada por sempre acreditarem que eu seria capaz de levar este trabalho até ao fim apesar dos momentos difíceis. Obrigada pelo apoio logístico, pelos incontáveis telefonemas, pelo orgulho e amor que eu sempre posso tomar como certos.

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ABBREVIATIONS AND SYMBOLS

α-CD	α-cyclodextrin
β-CD	β-cyclodextrin
γ-CD	γ-cyclodextrin
3-OMD	3-O-methyldopa
ACN	acetonitrile
ALAAD	aromatic L-amino acid decarboxylase
BBB	Blood Brain Barrier
br	broad
CD	cyclodextrin
CE	capillary electrophoresis
CEC	capillary electrochromatography
COMT	catecol-O-methyltransferase
CZE	capillary zone electrophoresis
d	doublet
DCM	dichloromethane
dd	doublet of doublets
DM-β-CD	dimethyl-β-cyclodextrin
DMSO	dimethylsulphoxide
DOPAC	3,4-dihydroxymethyl acetic acid
DOPAL	3,4-dihydroxymethyl acetaldehyde
Eq.	equivalent
GCMS	gas chromatography mass spectrophotometry
GI	gastrointestinal tract

HP-β-CD	hydroxypropyl β-cyclodextrin
HPLC	high performance liquid chromatography
i.d.	internal diameter
IPA	isopropanol
IR	Infrared
ITP	isotachophoresis
J	coupling constant
J_{gem}	geminal hydrogen's coupling constant
J_{vic}	coupling constant to a vicinal hydrogen
K_{obs}	observed pseudo-first-order reaction rate
LDEE	L-dopa ethyl ester
LDME	L-dopa methyl ester
L-dopa	3,4-dihydroxy-*L*-phenylalanine
LMWP	Low Molecular weight proteins
m	multiplet
M-β-CD	methyl-β-cyclodextrin
MAO	monoamino oxidase
MECC	micellar electrokinetic capillary chromatography
MEKC	micellar electrokinetic capillary chromatography
MeOH	methanol
MP	melting point
MS	mass spectrometry
NBS	N-bromosuccinimide
n.d.	not determined
NMR	nuclear magnetic resonance
NSAID	non-steroidal anti-inflammatory drugs
PD	Parkinson's disease
q	quartet
qi	quintet
$Rs_{x,y}$	resolution factor between peaks x and y
RT	retention time
s	singlet
S-β-CD	sulfated-β-cyclodextrin
SDS	sodium dodecyl sulfate
Sol.	solution
STDC	sodium taurodeoxylcholate

t	triplet
TBA	tetrabutylammonium phosphate sodium salt
TEA	triethylamine
TM-β-CD	trimethyl-β-cyclodextrin

CHAPTER 1. INTRODUCTION

1.1. Historical perspective

Several different approaches have been used, with more or less success, to tackle the problems of stability, formulation and delivery of drugs to the body. One of these approaches is the reversible derivatisation of the drug to an inactive form that has some preferable performance characteristics. At some stage of administration, absorption or distribution in the body, the derivative, which is called a *prodrug*, is converted back to the original drug.

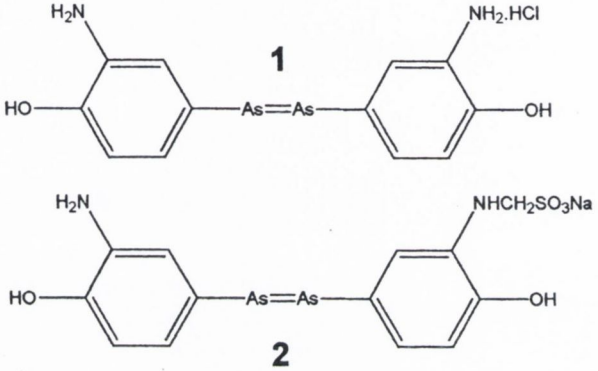
Prodrugs may also be prepared for other reasons, as will be described throughout this text, like the reduction of side effects, improvement of stability or patient acceptability of the drug.

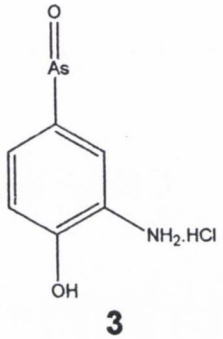
Many prodrugs were discovered accidentally and have been used for centuries in spite of their recognition as prodrugs being recent, like codeine for example, which is a prodrug for morphine[1].

It was Paul Ehrlich, at the beginning of the XXth century, who, in his search for the "magic bullet" that would kill bacteria but not other cells, initiated the era of synthetic chemotherapy and with it, the history of prodrugs.

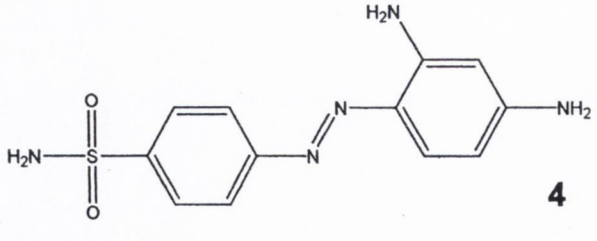
In 1909, the 606th compound of his systematic synthesis of atoxyl analogues, arsphenamine (**1**, Salvarsan), was found to be active against syphilis[2]. Salvarsan is an arsenic compound that is quite toxic and had the reputation of terminating the disease by eliminating the sufferer. Administration, which proceeded by intravenous injection for a period of over one hour, was also quite painful[3]. Neoarsphenamine (**2**), which was

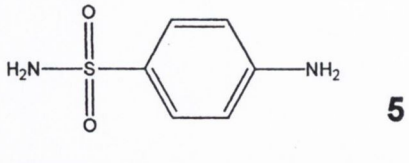
developed a few years later, was more water soluble and less toxic, but at the same time, much less active than Salvarsan[2]. The two drugs remained the main therapies for syphilis until the 1930s when it was found that the active compound was actually a metabolite, arsphenoxide (**3,** Mapharsen), which was safer[3]. Of course arsphenamine and neoarsphenamine did not have the characteristics desirable in prodrugs but, nevertheless, they were acting as prodrugs as they were converted to the active compound within the body.





Another remarkable story of an early prodrug is that of Prontosil (**4**), which was used for the treatment of streptococcal infections. Prontosil is a prodrug of sulphanilamide (**5**) and it was developed by Gerhard Domagk in 1932 for, gossip says, commercial reasons rather than pharmacokinetic ones, because the active compound was not patentable. It was a French group at the Pasteur Institute that speculated that the azo link might not be necessary for therapeutic efficacy and that the active principle could be formed by reduction of the azo bond. They later proved that the antibacterial activity resided in the sufanilamide portion of the molecule[4].





Despite these early developments, the term *prodrug* was used for the first time only in 1951[5] and active attempts to produce prodrugs started only about 35 years ago. Since then, research on prodrugs has been increasing steadily. An on-line yearly search on

Science Direct (Elsevier) database illustrates (Figure 1.1), by means of the number of publications on the subject, how important this kind of approach has become recently.

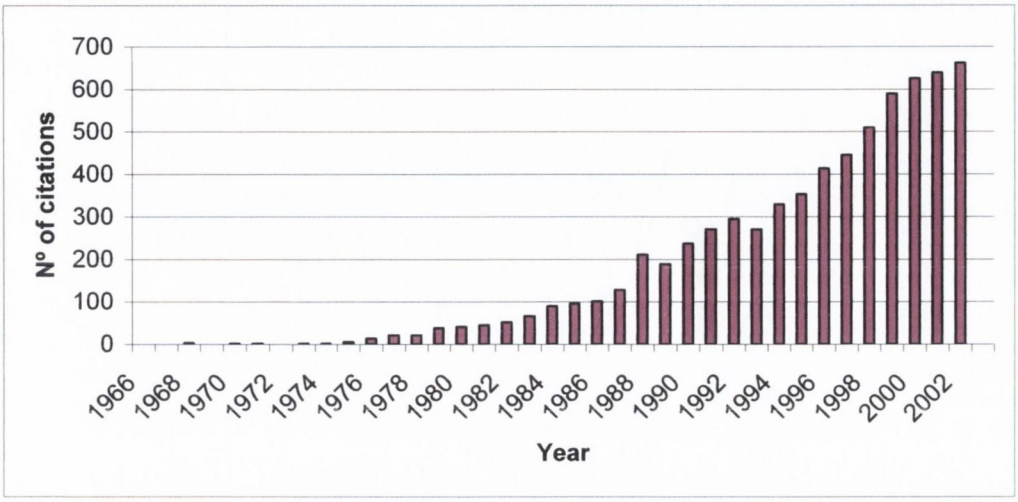


Figure 1.1: Number of publications on prodrugs according to an on-line search on a scientific database

This chapter will provide an overview of the different ways of improving the pharmacokinetic properties of drugs by producing prodrugs. Since esterification is the approach with the largest number of applications and the highest rate of success, a part of this text will be devoted to it. However, not all drugs have chemical functions amenable to esterification and quite often other solutions have to be found. Amines are one example where other approaches have been taken. Nevertheless, no development has led to a generally applicable solution. Due to the relevance to the rest of this work, those approaches will be reviewed.

1.2. Overview to prodrug systems

1.2.1. The prodrug concept

By definition, a prodrug is a pharmacologically inactive derivative of a drug molecule that, within the body, predictably transforms releasing the active drug[6].

Prodrugs have been developed in order to overcome pharmaceutical and/or pharmacokinetic based problems associated with the parent drug molecule. These problems may be related to the drug absorption and distribution, but also to its stability, toxicity and side effects.

Most times, prodrug design aims to increase the therapeutic index (TI) of the drug, which is the ratio between activity and toxicity and reflects the margin of safety of the drug[7].

Usually, only a small part of the administered dose of a drug reaches the site of action either due to difficulties in its absorption and distribution or because the drug is metabolised before reaching its target.

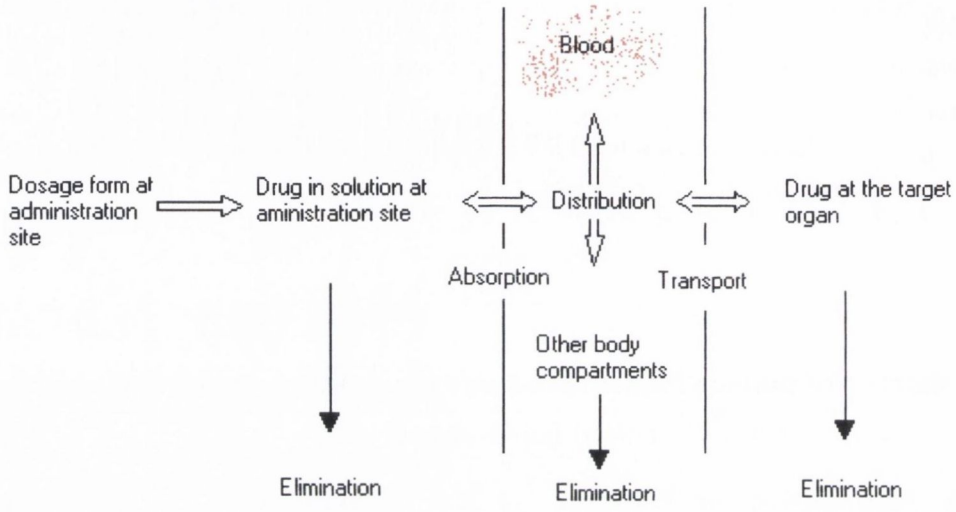


Figure 1.2: Fate of drugs in the body (adapted from[8])

Sometimes it is sufficient to manipulate dosage forms in order to improve dissolution rates or overcome metabolism or chemical degradation before absorption. On the other hand, avoiding metabolism after absorption or improving passage through membranes and delivery to specific sites in the body, may need a bioreversible chemical derivatisation of the drug, or prodrug preparation. After administration, prodrug to drug conversion should occur as soon as the desired goal for designing the prodrug has been achieved. This may be before, during or after absorption or at a specific site in the body[6].

Prodrug systems bear some resemblance to protecting groups in organic synthesis[9]. Nevertheless, not all the conditions that can be applied *in vitro* to remove protecting groups or produce structural changes, are achievable within the body and therefore, most commonly used strategies in organic synthesis can not be applied in prodrugs.

Equally, prodrug designs can avail of biological systems for unmasking the drug, which are not available to the organic chemist or are not practical for use in the synthetic laboratory (as the P450 enzymatic system, for example).

1.2.2. Rationales for drug derivatisation

Improving bioavailability and membrane passage is one of the main areas of prodrug development and there are several ways of achieving these objectives through manipulating the physical and chemical characteristics of the drug.

Formation of prodrugs to **improve aqueous solubility**[10,11,12] is common. Low aqueous solubility limits the administration in the form of injectables and gives rise to dissolution rate dependent oral bioavailability. On the other hand, sometimes it is necessary to **alter the lipophilicity**[8] of an aqueous soluble drug to allow its solubility in the lipidic layers of biological membranes. Nevertheless, aqueous solubility is also necessary for the drug to get out of the membrane. Usually a compromise between aqueous solubility and lipophilicity has to be achieved for maximum bioavailability. Particularly in the case of dermal absorption these two properties have to be carefully adjusted since biphasic solubility is a determinant of the flux across the skin[8].

Typically, ionised species, while more soluble in aqueous solutions, do not pass several body barriers by passive diffusion as they are not soluble in the lipidic material. The pH partition theory[8,13] allows the prediction of the extent of drug absorption based on the pH of the intestinal tract and the pK_a of the drug (Figure 1.3).

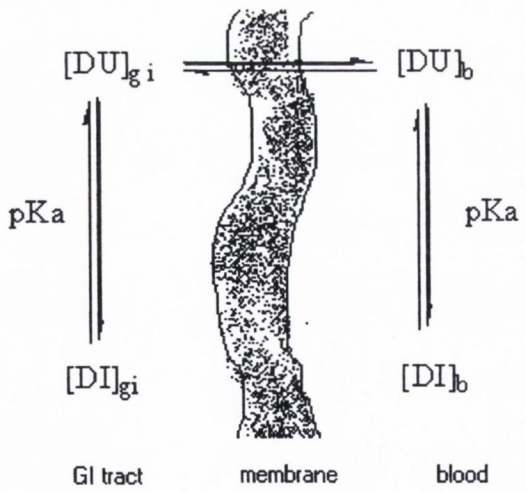


Figure 1.3: Absorption of a drug according to its pK_a

The ratio *D* between the total concentration of the drug in the blood and in the gastrointestinal (GI) tract can be calculated by (**1.1**)

$$D = \frac{[DU]_b + [DI]_b}{[DU]_{gi} + [DI]_{gi}} \qquad \textbf{(1.1)}$$

where $[DU]_b$, $[DI]_b$, $[DU]_{gi}$, $[DI]_{gi}$ represent the concentration of the drug, in unionised and ionised forms, in the blood and in the GI tract respectively.

The ratio between the ionised and non ionised forms is a function of the pH of the solution and of the pK_a of the drug as described by the Henderson-Hasselbach equation (**1.2**) (example for a weak base, activity not considered):

$$pK_a - pH = \log\frac{[DI]}{[DU]} \qquad \textbf{(1.2)}$$

Admitting that only the non ionised form of the drug passes through the membrane and that the transfer stops when $[DU]_{gi}=[DU]_b=1$, for a weak base of $pK_a=5.4$ in blood (pH=7.4):

$$\frac{[DI]_b}{[DU]_b} = 10^{pKa-pH} = 0.01 \qquad \textbf{(1.3)}$$

while in the duodenum (pH≈6.4):

$$\frac{[DI]_{gi}}{[DU]_{gi}} = 10^{pKa-pH} = 0.1 \qquad \textbf{(1.4)}$$

D can finally be calculated:

$$D = \frac{1+0.01}{1+0.1} = 0.92 \qquad \textbf{(1.5)}$$

which means that the total concentration of the drug in the blood (after equilibrium is reached) is about 92% of the concentration of the drug in the duodenum.

The same calculations applied to a stronger base ($pK_a=8.4$), would lead to a *D* ratio of 0.11. This means that, a weak base can be expected to be significantly better absorbed than a stronger one, for comparable lipophilicities. Also, weak acids are expected to be better absorbed than stronger acids.

Since the pH in the stomach (pH<3) is significantly lower than in the duodenum, the absorption of acids is favoured in the stomach. Nevertheless, one must bear in mind that the area available in the rest of the GI tract is much larger, which may account for a higher percentage of the drug being absorbed there anyway. On the other hand, the absorption of bases is not favoured in the stomach as these are ionised there.

This approach can only be applied to drugs with suitable lipophilicities since, between the GI tract and the blood, there is a barrier that has to be permeable to the compound. Lipophilicities are usually estimated based on the octanol / water partition coefficient of the drug. When assessing the partition coefficient of a compound at a particular pH, the extent

of ionisation has to be taken into account, as the partition coefficient of an ionic species is usually negligible. The partition coefficient (P_{pH}) of a weak base at a given pH can be expressed by

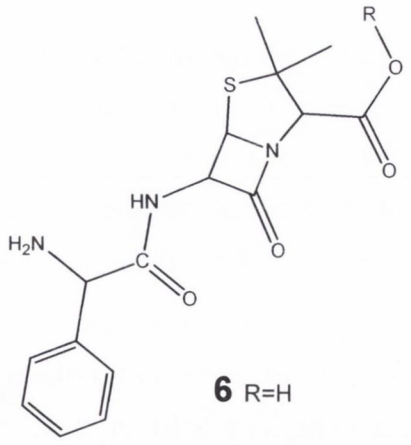
$$P_{pH} = P_B f_B \qquad \textbf{(1.6)}$$

where P_B is the intrinsic partition coefficient of the base and f_B is the fraction of uncharged species at the given pH. Taking into account the Henderson-Hasselbach relationship (**1.2**), the equation can be rewritten as

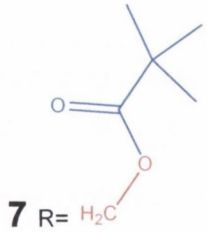
$$\log P_{pH} = \log P_B - \log(1 + 10^{(\mathrm{pKa-pH})}) \qquad \textbf{(1.7)}$$

which shows that the passage across a membrane is dependent on the intrinsic lipophilicity of the drug as well as on its pK_a. The design of a prodrug may, for this reason, involve a modification of the pK_a. Once a molecule has an adequate pK_a, a log P of about 2 is usually considered optimal for intestinal absorption as long as dissolution is not rate limiting[8].

Esters may be used as prodrugs of carboxylic acids to favour intestinal mucosa passage by quenching the ionic moiety. One of the better known examples is ampicillin (**6**): only 30% to 50% of the administered oral dosage is absorbed if the carboxylic acid form is used, but better absorption may be achieved if double ester prodrugs (pivampicillin (**7**) and bacampicillin (**8**)[14]) are used[8] (the esterase sensitive progroup is in blue and the group eliminated spontaneously is in red; this convention is used throughout the rest of this work).



6 R=H



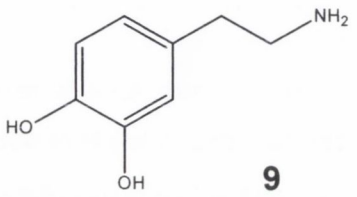
7 R= H_2C

8 R= HC

These compounds have increased acid stability and give higher plasma peak levels of ampicillin than underivatised ampicillin. The cumulative percentage absorbed, of a given dose, after passing through the stomach, the duodenum and the jejunum of volunteers receiving oral aqueous solutions of the test compounds, was 71% for bacampicillin and 31% for ampicillin. This improved absorption can be attributed not only to the increased lipophilicity of the prodrugs but also to the higher solubility in aqueous solutions[13].

Passage through membranes may also be optimised by targeting active carriers[15] like the intestinal peptide transporters. In this approach, the prodrug is designed in the form of a di- or tripetide analog of the drug.

L-dopa (**10**) is a prodrug of dopamine (**9**) used for Parkinson's disease. Administered on its own, dopamine does not reach the target organ, the brain, as it is too polar to pass the blood brain barrier (BBB). L-dopa, while also being polar and not passing the BBB through passive diffusion, can use the amino-acid transport system to the brain where it is then decarboxylated by L-aromatic amino acid decarboxylase (ALAAD) releasing dopamine at its site of action[16].



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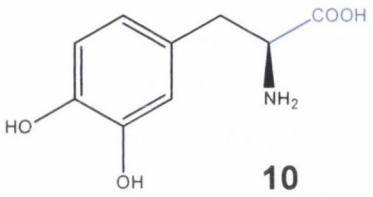

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Improving the bioavailability of a drug by means of a prodrug, may allow a significant reduction in the administered dose with consequent benefits in terms of **the reduction of side effects and toxicity**. Reducing the side effects in terms of gastric mucosa irritation while maintaining water solubility, is the main goal in the attempts to produce prodrugs for aspirin[17] and ibuprofen[18].

One area currently under intense research is **site specific drug delivery** or targeted drug delivery[19,20].

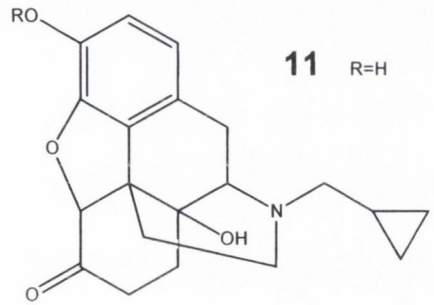
Targeting is especially attractive for highly toxic drugs having a narrow therapeutic window, particularly those used in cancer therapy. In this approach, it is more important to increase the ratio of the concentration of the drug in the target organ to the concentration in non-target organs, than it is to increase the bioavailability throughout the whole body[19]. Several examples of prodrugs developed for site specific targeting will be given in section 1.2.5.

Another situation where the preparation of a prodrug is often necessary to increase its bioavailability, is when the drug is subjected to extensive **first pass metabolism**, which not only reduces the bioavailability of the drug, but may also lead to the formation of metabolites, which can cause adverse effects. First pass metabolism consists in the pre systemic inactivation of the drug. It can occur in the intestine or in the liver. While it can be avoided by using routes of administration other than oral, this is not practical most times[6].

Molecules having phenolic hydroxyl groups are one class of compounds where first pass metabolism is most important. Dopamine (**9**) and its prodrug L-dopa (**10**) are examples of this situation as the catechol group is easily metabolised by sulfuration, glucuronidation or

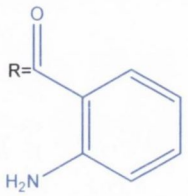
methylation with catalysis by enzymes present in the gut and liver like catechol-O-methyltransferase (COMT). Moreover, L-dopa can also be decarboxylated to dopamine even before being absorbed or reaching the brain. Several attempts have been made to produce prodrugs for dopamine or L-dopa as will be described later (Chapter 5), but to date L-dopa still remains the most effective one[21].

Some phenolic drugs have benefited from protection of the hydroxyl group, like naltrexone (**11**) whose anthranilate (**12**) and salicylate (**13**) esters enhanced the bioavailability 45 and 28 fold, respectively, relative to the drug[6,8].



11 R=H

12



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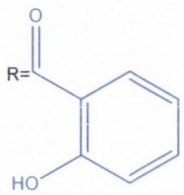
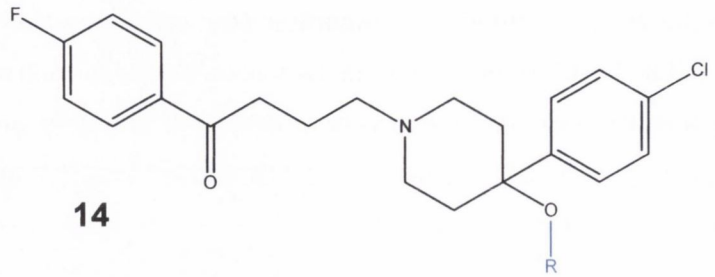


Depressing first pass metabolism can also be achieved by incorporating an enzyme inhibiting function or by derivatising the drug molecule at another position so that the prodrug is no longer a substrate for the metabolising enzyme[8].

Prolonging the duration of action of a drug may be desirable, particularly in chronic diseases. With slow and prolonged release drugs, the number and frequency of the doses required can be reduced and the levels of drug in circulation are more stable. The peak level of the drug in the blood may also be reduced, reducing as well the possibility of dose related toxicity and side effects.

One way of achieving this, consists in producing slow release formulations which deliver the drug slowly to the systemic circulation, but even in this case some modification of the drug might be necessary. The concept has been applied to contraceptive steroids that, in the form of highly lipophilic esters are released very slowly from an oil vehicle administered via intramuscular route. After entering the blood stream the esters are rapidly hydrolysed.

The same principle was applied to haloperidol (**14**, R=H) whose decanoate ester (**14**, $R=CO(CH_2)_8CH_3$) can be administered as a solution in sesame oil from which the prodrug is released over about a month[22].



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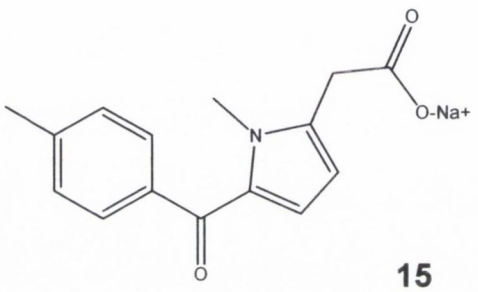
Another possibility consists in preparing prodrugs that possess a slow conversion to the parent drug in the organism. The tolmetin-glycine (**16**) conjugate has a peak concentration of about 9 hours while, with the unconjugated drug tolmetin sodium (**15**), the peak concentration is observed after one hour[6].



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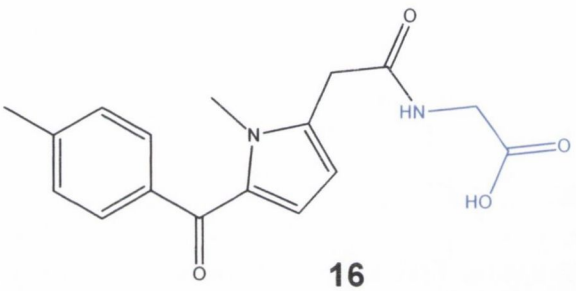

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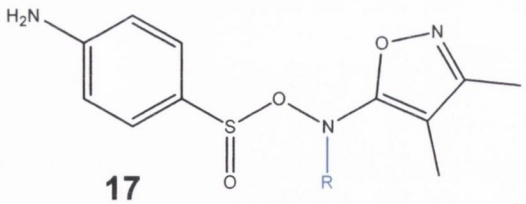
Poor patient acceptability is another situation where the preparation of a prodrug might be advantageous. An active drug may have an unpleasant taste or odour or it can cause gastric irritability or pain when administered (injectables), and a reversible alteration of its structure may alleviate these problems. One example is the anti bacterial sulfisoxazole (**17** R=H) which has a bitter taste, while sulfisoxazole acetyl (**17** R=$COCH_3$) is tasteless[6].



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Improvement of drug formulation might justify the development of a prodrug. For example it might be necessary to produce stable derivatives of unstable drugs and allow preparations with longer shelf lives.

1.2.3. Mechanisms of activation of prodrugs

The prodrug approach has also been called *drug latentation* and it can be further refined into two classes: carrier linked prodrugs and bioprecursors[6].

Carrier linked prodrugs are the most common. In this type of prodrug, a progroup or carrier, is linked to the active drug by an *in vivo* labile bond from which the drug is released by

hydrolysis or other type of cleavage. A bioprecursor, on the other hand, cannot be converted to the active drug by simple cleavage; it has to be metabolised by molecular modification into a new compound that is the active drug[6].

The conversion or activation of carrier linked prodrugs can be achieved by means of a chemical or enzymatic reaction. Some chemical reactions usually happen upon a change in the pH of the surrounding medium. For example, different pHs are encountered across the GI tract and between the GI tract and the blood. Certain prodrugs are designed to be stable in the pH range 3-5 but susceptible to hydrolysis at blood pH. On the other hand, some compounds, in spite of being stable at a wide range of pHs, can be converted by the action of one or more of the many enzymes available within the body. Esterases are the most common example but many other enzymes like peptidases, glutamases and decarboxylases have been used with more or less success to convert prodrugs to the corresponding drugs.

Examples of bioprecursors are scarcer than examples of carrier linked prodrugs but this is the principle underpinning, for example, prodrugs that use redox systems as means of activation.

Site specific bioactivation *via* reductive systems has been studied for example for tumour therapy and is receiving increasingly attention[23]. These systems make use of tumour hypoxia to initiate a sequence of reduction reactions that ultimately release the original drug. These types of reductive systems, may or may not be assisted by endogenous enzymes and will be further discussed as applied on prodrug systems for amines (in section 1.3). Another possibility of activation of these types of prodrugs is via therapeutic radiation[23].

1.2.4. Esters and double esters as prodrugs

Esters are probably the most common type of prodrug and are used for drugs containing carboxyl or hydroxyl functions. This is because of the large number of esterases existing in the organism that are able to hydrolyse these compounds, and also because, depending on the type of ester used, it is possible to produce derivatives with the desired characteristics[24]. In addition to this, non-enzymatic hydrolysis may also occur[8,20] in some cases.

Different species usually display marked differences in the *in vivo* hydrolysis of prodrugs[20]. One example is thiazolidine carboxyl group whose simple alkyl and aryl esters are rapidly hydrolysed to the free penicillin acid in rodents but are much more stable in man.

Nevertheless the acyloxymethyl ester (a double ester - or a tripartate prodrug) is rapidly hydrolysed in man, the reason being that penicillin esters are highly hindered while the terminal group of the double ester is less hindered[20]. Pivampicillin (**7**), a prodrug of ampicillin (**6**) is, as mentioned earlier (page 7), another successful example of an acyloxymethyl ester prodrug.

The general mechanism of release of the drug in acyloxyalkyl esters can be illustrated by yet another example which is the pivaloyloxyethyl ester of methyldopa (**18**, Figure 1.4)[20]. The release of the drug is thought to happen after two steps: the first step consists in an enzymatic reaction while the second one is spontaneous.

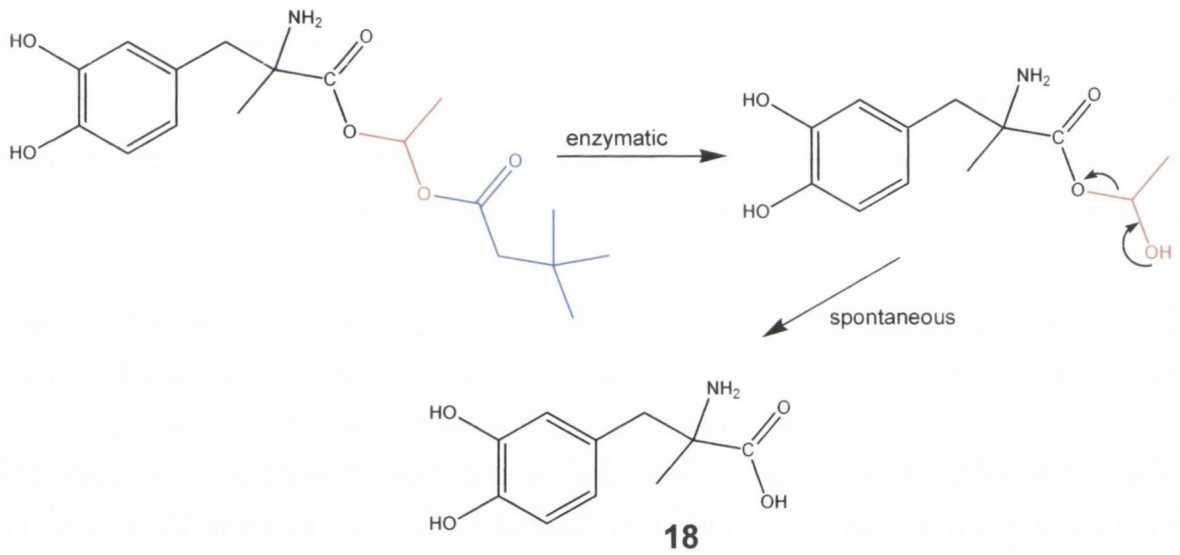


Figure 1.4: Mechanism of cleavage of acyloxyalkyl esters

Esters containing a carbonate structure, where the mechanism of release of the drug is the same as the one described above, have also been prepared and commercialised like in the case of bacampicillin (**8**)[14].

Acyloxyalkyl carbamates have been proposed as prodrugs for amines (cf. page 26) but they can also be seen as a means of preparation of prodrugs for carboxylic acids as these compounds are generated during the first cleavage of the prodrug.

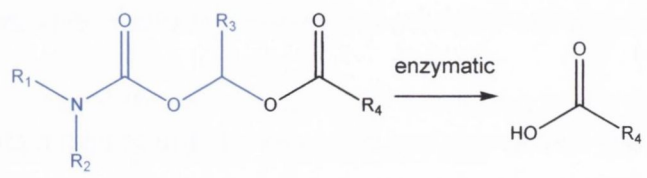


Figure 1.5: Acyloxyalkyl carbamates as prodrugs for carboxylic acids

The concept has been applied to non-steroidal anti-inflammatory drugs (NSAID) as an attempt to produce prodrugs for dermal delivery with good *in vitro* stability. Nevertheless the results were disappointing, as skin permeation was poor[25].

Although all the examples shown are applications of the ester prodrug concept to protect carboxylic drugs, esters can also be used as a means of protection of hydroxyl containing drugs. Phenolic compounds, which undergo extensive first pass metabolism, have been protected by means of O-α-acyloxyalkyl esters which, albeit being chemically stable, are enzymatically hydrolysed to an unstable hemiacetal intermediate that is afterwards spontaneously converted to the phenol[26]. Phenyl carbamates have also been suggested as prodrugs for phenols[27,28].

Amides can also benefit from the preparation of an ester in the form of a tripartate prodrug. N-acyloxyalkylation of NH-acidic compounds (Figure 1.6) can be used to produce prodrugs amenable to enzymatic hydrolysis and further breakdown to the original drug.

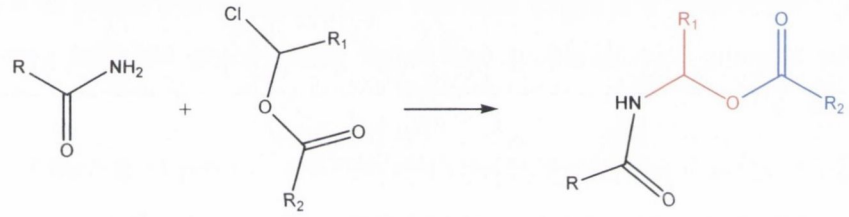
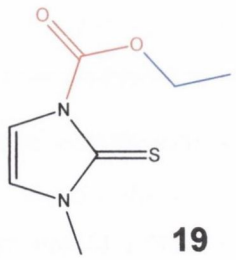


Figure 1.6: N-acyloxyalkyl derivatives of amides

Enzymatic hydrolysis produces the N-hydroxyalkyl derivative (which is, *per se*, a possible approach to produce prodrugs for amides) that then breaks to the original drug spontaneously[20,29].

The system is mostly used with acyloxymethyl compounds, which release formaldehyde spontaneously, from the intermediate formed by enzymatic hydrolysis. Manipulation of the acyl moiety enables the production of compounds with the desired solubility characteristics[8]. This double prodrug approach has been applied, for example to 6-mercaptopurine, by combining acyloxymethylation with Mannich derivatisation, which improved the permeation rates[30].

N-Acyl derivatives of amides and imides, have also been used to produce compounds sensitive to esterases, with improved absorption when compared to the parent drug. For these compounds, enzymatic assisted hydrolysis is usually higher than pH dependent hydrolysis, which enables the improved absorption of the prodrug, prior to the release of the drug[31]. Carbimazole (**19**), a prodrug of the antithyroid methimazole is cleaved to the active drug in the presence of serum enzymes[8].



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1.2.5. Targeted delivery and activation

Historically, prodrugs have been developed to circumvent some kind of biological obstacle in order to improve its systemic delivery, with the belief that elevated systemic drug levels translate into increased levels at the response site and, consequently, into an intensified pharmacological response. However this is not always a feasible approach because, by increasing the systemic levels of a drug, it is probable that an increase in its side effects also happens. This is why, the ability to attain high local concentrations of a drug through targeting, while keeping low levels at non-target sites, could alleviate potentially dose-limiting side-effects[19].

Site specific drug delivery includes not only the delivery of drug to a particular organ (site directed/selected drug delivery or site specific transport[19]) but also the specific activation of the drug in the target organ. Site specific transport, using antibodies as carriers for drugs or for enzymes[32] that convert a prodrug to the active compound, is currently under intense investigation[19].

Site specific-bioactivation exploits the fact that there are chemical differences between the target site and other sites that may enhance the conversion of the prodrug to the drug.

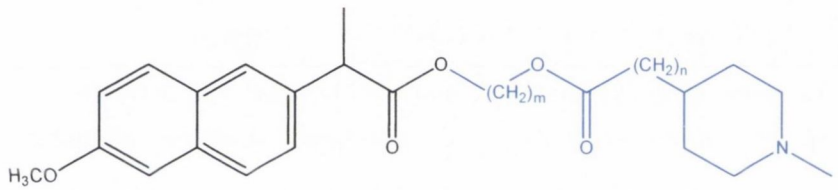
1.2.5.1. Prodrugs for topical administration

One apparently simple way of achieving targeted delivery, is the administration of the drug directly on the site of action, for example, on a bruise on the skin or in the eye. Nevertheless, because the skin and the cornea are membranes "designed" to protect the body from foreign materials, it may be necessary to provide drugs with particular characteristics to overcome them. On the other hand, in some cases, these barriers may actually be easier to overcome than others, like the intestinal membrane, providing alternative means of delivering drugs to the systemic circulation. Examples of these two situations will be given.

a) Prodrugs for dermal delivery

As mentioned before, when developing drugs for dermal delivery, a correct balance between aqueous solubility and lipophilicity has to be achieved.

Naproxen is a non-steroid anti-inflammatory drug (NSAID) used for the treatment of rheumatic diseases, and its bioavailability, when topically applied, is only 1-2%. The possibility of using esterification of the carboxyl group as a means of increasing lipophilicity and consequently improving dermal absorption has been studied[33,34]. Although simple esters are better absorbed, they release the drug too slowly. On the other hand, naproxen acyloxyalkyl esters, that make use of the double prodrug concept (section 1.2.4), are hydrolysed at rates that are suitable for their application as prodrugs[34]. Some methylpiperazinyl acyloxyalkyl prodrugs (**20**) combine good biphasic solubility and fast enzymatic hydrolysis and give improved topical delivery of naproxen[33].



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1-Alkylazacycloalkan-2-one esters of ketoprofen have also been prepared as a means of increasing lipophilicity. Some of them showed better cumulative absorption through the skin and some sustained activity[35].

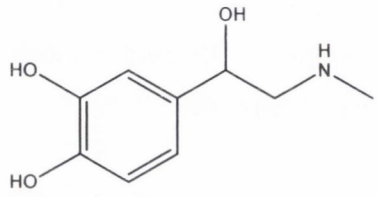
Naltrexone (**11**) is an opioid antagonist used for treatment of narcotic dependence and alcoholism. Its oil-soluble alkyl ester prodrugs are 2-7 fold better absorbed than the phenolic form and show a significant metabolic conversion to the active drug in the skin. These provide promising alternatives to the original treatment, with better chances of patient compliance[36].

b) Prodrugs for ocular delivery

Although most ocular diseases are treated by topical application of eyedrops, typically less than 5% of the applied dose reaches the intraocular tissues. The basis of prodrug design for improved drug delivery to the eye, resides in preparing compounds with increased corneal passive diffusion so that the ratio (distribution to the eye)/(systemic distribution) is increased. Many ocular drugs can be derivatised to esters which are more lipophilic and

consequently better absorbed; since ocular tissues contain butyrylcholinesterase and acetylcholinesterase while tears do not, hydrolysis of the prodrugs occurs mostly after absorption. Nevertheless, this approach is not deprived of problems like stability and solubility (in eyedrop formulations) as well as irritation, which may be significant obstacles to its application, such as in the case of some pilocarpine mono and diesters[37].

The first prodrug applied in ophthalmology was dipivefrine (**22**), a dipivalyl-ester prodrug of epinephrine (**21**) with significantly better ocular absorption.



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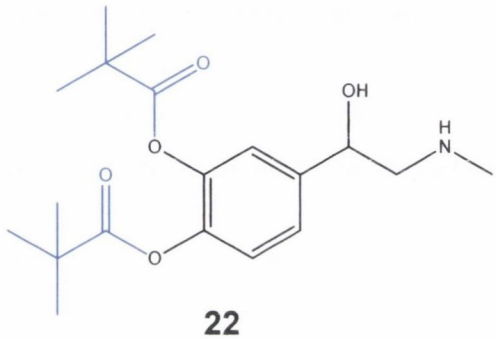

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Although the fractions of dipivefrine and epinephrine that are absorbed systemically are similar, the use of the prodrug results in a smaller systemic availability of the drug (consequently with less side effects) because smaller doses of dipivefrine can be applied.

Many other examples could be given of this kind of approach and ref. 37 provides a good review, but many attempts did not result in commercial prodrugs.

Ester prodrugs of steroids have been prepared and commercialised. However, there is no correlation between permeability through the cornea and their efficacy, which has raised the question of their intrinsic activity as opposed to their benefiting from the prodrug approach[37].

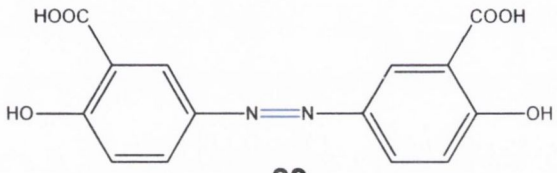
c) Prodrugs for nasal delivery

Nasal delivery is an alternative to other routes of administration with some advantages including high systemic availability. Both charged and uncharged forms can be transported across the nasal epithelium thus providing a more patient-acceptable alternative to parenteral administration. Since this mucosa is rich in several metabolising enzymes, transporters and receptors, it is possible to envisage the development of prodrugs targeting them[38]. Some recent developments involve prodrugs aimed at the CNS and anticancer drugs[39].

1.2.5.2. Prodrugs for colon activation

One of the basic principles behind colonic targeting is the preparation of prodrugs that, due to their high polarity, have very poor pre-colonic absorption but, once in the colon, are converted to the active drug[19], which is absorbed there.

A classic example of site specific-transport and bioactivation is olsalazine (**23**), a prodrug of 5-aminosalicylic acid that breaks into two molecules of the drug in the colon by the action of azo-reductases secreted from colonic bacteria, while passing unaffected through the intestine where it is poorly absorbed[8,20].



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Other azo compound have also been prepared like sulfalazine and balsalzide which link a molecule of 5-aminosalicylic acid to molecules of sulfapyridine and 4-aminobenzoyl-β-alanine respectively, thus providing alternative treatments[40].

Glycosidic and glucurinidic prodrugs, particularly from corticosteroids, that exploit bacterial glucosidases and glucuronidases have also been tested with promising results[41].

More recently, amino acid and glutamic derivatives of 5-aminosalicylic acid were also tested. 5-aminosalicy-L-aspartic acid was effectively delivered to the large intestine releasing about half of the administered dose of 5-aminosalicylic acid[42].

1.2.5.3. Prodrugs for kidney activation

Two approaches of drug delivery to the kidney have been preferentially studied: the prodrug and the low-molecular weight protein (LMWP) approaches. Most research has focused on prodrugs that require activation by kidney selective enzymes[43]. More that 25 years ago it was realised that it might be possible to prepare prodrugs targeting the kidney, based on the fact that this organ possesses high concentrations of γ-glutamyl transpeptidase, an enzyme capable of cleaving γ-L-glutamyl derivatives of aminoacids and peptides[8,44].

For example, when dopamine is intravenously administered in the form of the double prodrug γ-glutamyl-L-dopa (gludopa), it leads to kidney levels of the drug that are about five fold higher than the ones obtained with equimolar quantities of the single prodrug L-dopa[19]. Nevertheless, poor bioavailability rules out gludopa as an oral dopaminergic prodrug[45].

N-acetyl-L-γ-glutamyl derivatives of some drugs were also evaluated. Studies on prodrugs of model drugs showed that some resulted in effective delivery to the kidney while others did not, thus showing that the concept is not universally applicable. N-acetyl-L-γ-glutamyl p-nitroanilin and N-acetyl-L-γ-glutamyl aminophenyl acetic acid showed selective uptake by the kidney[46], but in the case of 4'-aminowarfarin, although the prodrug is a substrate for the enzyme, there was no evidence of selectivity to the kidney in rats[47].

Alkylglycoside prodrugs were also shown to be suitable candidates as vectors for renal targeting[48]. Sugar moieties have been suggested for the delivery of low molecular weight peptides[49].

Difficulties in transporting drugs to the kidney may be overcome by attaching them to small proteins that are catabolized in the lysosomes of the proximal tubular cell[43,50]. These systems have been applied to carboxylic and amine drugs, with or without a spacer between the drug and the LMWP[51]. Lysozyme conjugates of naproxen and captopril, connected to the protein via the peptide bond have resulted, after a bolus dose was administered, in increased concentration of the drugs in the kidney and lower concentration in the plasma, when compared to equivalent doses of the free drug[52,53].

1.2.5.4. Targeting viruses

The main approach in targeting viruses has been to use enzymes produced by them to activate drugs that cause their own destruction.

The antivirals acyclovir (**24**), pencyclovir and famcyclovir are all bioprecursor prodrugs that are used in the treatment of herpes and are converted to the active triphosphate ester by the successive action of virus-encoded kinases and cellular enzymes[54], mostly from infected cells (Figure 1.7). Because of its site-specific activation these drugs have very little toxicity against uninfected cells.

Pencyclovir, which has a slightly different side chain, is much more active than acyclovir but it has very poor oral bioavailability and therefore a pencyclovir prodrug, famcyclovir was commercially developed. This new prodrug has good oral bioavailability and shows almost complete bioconversion to pencyclovir[19].

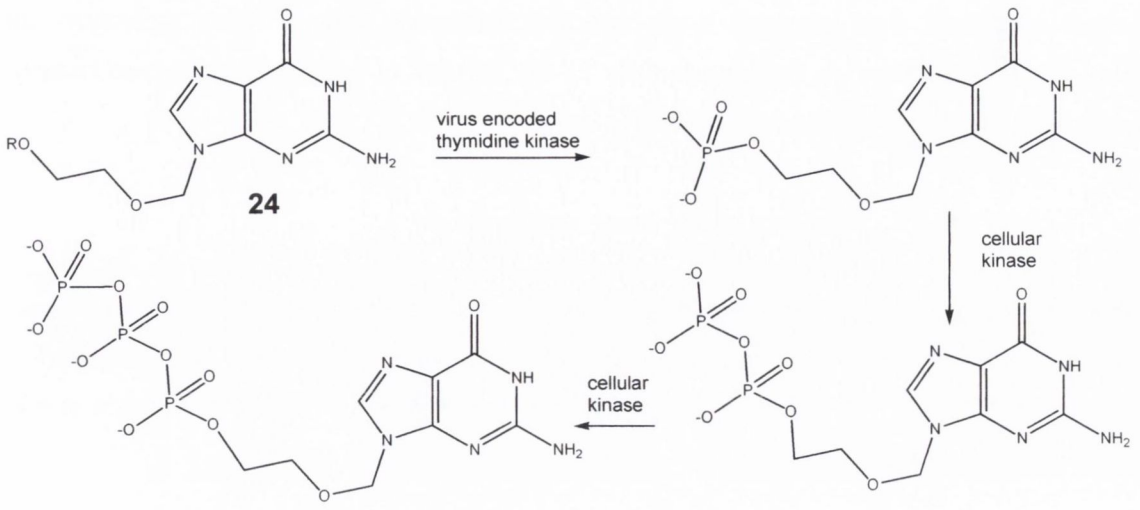


Figure 1.7: Mechanism of activation of the prodrug acyclovir

1.2.5.5. Prodrugs for cancer therapy

The majority of clinically used anticancer drugs are anti-proliferative agents (cytotoxins) that somehow preferentially kill rapidly dividing cells. However, these agents are not always selective for cancer cells and thus, usually have high toxicity to proliferating normal cells[23].

A chance of diminishing this toxicity relies on producing prodrugs that are delivered preferentially or are selectively activated in the tumour and not in other parts of the body. Denny[23] provides a good review from where, a few examples described here were taken.

The fact that some tumours are surrounded by high concentrations of nitric oxide can be exploited[55]. Reductive pathways, that rely on the increased proportion of hypoxic cells in tumours as means of activating prodrugs (like the one described in page 33), are also under intensive investigation[56]. Other aspects of tumour physiology that can be used for prodrug activation include selective enzyme expression and low extracellular pH.

Another possibility is the activation of prodrugs by exogenous enzymes that are delivered to the tumour cells via monoclonal antibodies, in a technique that is called antibody-directed enzyme-prodrug therapy (ADEPT). An alternative concept is gene-directed enzyme-prodrug therapy (GDEPT) in which the gene, that codifies an enzyme capable of activating a prodrug, is introduced in the genome of the tumour.

Prodrugs have also been prepared by linking cytotoxins to tumour specific antibodies. The linkage is cleaved, following uptake of the conjugates by endocytosis. The concept has been applied to Doxorubicin amongst others and has reached phase II trials.

A new approach that involves both specific transport and specific activation uses oligonucleotide sequences complementary to the mRNA of proteins expressed mainly in tumours, to carry and activate the drug[57].

1.2.5.6. Prodrugs for liver activation

Targeted delivery to the liver has been attempted by exploiting site selective transport pathways like the bile acid transport system and asiaglycoprotein receptor-mediated endocytosis[19]. Since the liver is a prime site for metabolism of drugs, the potential for its use for prodrug activation is high and varied.

Prodrugs were tested for improved delivery of d-α-tocopherol to the liver. The prodrugs are acid salts of aminoalkene carboxylic acid esters of d-α-tocopherol that, after *iv* administration, are selectively uptaken by the liver and metabolised by liver esterases. They have preventive efficacy against liver oxidative injury associated with free radicals[58].

4-(2,2-Dimethylethyl)-2-(4-methylphenyl)[1,3]dioxolane has been found to reduce glucose levels in animal models of diabetes by reducing fatty acid oxidation and hence depriving the system of the energy and cofactors necessary for gluconeogenesis. Nevertheless, the drug has toxic effects in other organs at the levels needed for therapeutic effectiveness. For this reason, an approach was developed, that utilises the natural processing of trigliceride-like intermediates as a basis for selectively targeting absorption, processing and delivery of a prodrug to the liver without releasing toxic amounts of the drug into circulation[59].

Another study investigated the ability of N,N-dimethylglycine esters of menahydroquinone to undergo cleavage to the parent drug, menahydroquinone-4, by a human tissue catalysed hydrolytic pathway[60].

Prodrugs of the aforementioned pencyclovir and other antivirals, which rely on an oxidative reaction catalysed by cytochrome P450 isoenzyme for activation, are currently under clinical testing. The prodrugs are stable in other tissues but undergo efficient and specific activation to the active triphosphate in the liver[61].

1.2.5.7. Prodrugs for CNS delivery

The blood brain barrier is both a transport and an enzymatic barrier. Tight junctions between cells in brain capillaries are an effective barrier against passive diffusion while a number of metabolic enzymes within capillary endothelial cells, effectively impede the passage of several exogenous compounds. Some of those enzymes are aromatic L-amino

acid decarboxylase (ALAAD), catechol-O-methyltransferase (COMT) and monoamine oxidase (MAO). Many others could be mentioned and, although they provide protection by excluding exogenous compounds from reaching the brain, they can also be used to selectively carry out prodrug conversion[62]. In addition to these protection mechanisms, there is also an effective efflux mechanism provided by P-glycoprotein which selectively pumps some compounds out of the brain even if they have adequate lipophilicities to overcome this barrier by passive diffusion[63].

On the other hand, there are some active transporters for biological anionic, cationic and peptide molecules that can be used also to carry drugs into the brain[15]. Examples of such transporters are the large neutral amino acid transporter and the monocarboxylic acid carrier. L-dopa is one compound that takes advantage of the large neutral amino acid transporter to deliver dopamine to the brain.

Several prodrugs have been developed to enhance brain penetration, mostly by manipulating its lipophilic character as a way of improving their passage through BBB lipidic bilayers. However this approach does not constitute *per se* a site-specific approach as the concentration of the drug in the brain is not preferably increased in comparison with other lipophilic sites in the body. Nevertheless, lipophilic esters of highly polar drugs can effectively increase the proportion of drug in the brain, as the free drugs become locked in the brain as a result of their polarity.

Adenosine deaminase exists in higher levels in the brain than in the blood and attempts have been made to use it as a targeting vector for brain-specific delivery. The concept has been applied to certain anti-HIV agents and other antivirals, with improved brain/plasma level ratios. However, the fact that this enzyme also exists in large quantities in spleen and intestinal tissues, represents a potential impediment to the clinical application of this system[62].

An example of brain targeting, is the dihydropyridine⇄pyridinium salt interconversion redox system, developed to transport and trap drugs in the brain (Figure 1.8)[64,65].

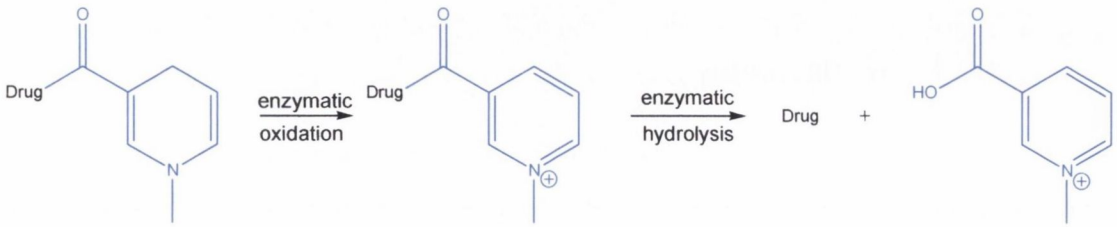


Figure 1.8: Redox carrier system to the brain

The lipophilic prodrug penetrates the BBB and the salt obtained after oxidation is trapped in the brain where it slowly releases the drug. This system is applicable to different functional groups like amines, alcohols and carboxylic acids[66,67]. Any oxidation in peripheral compartments results in rapid elimination of the intermediate due to its high polarity. This way, the drug can be preferentially accumulated in the brain. The major limitation to this system is the facile oxidation of the dihydropyridine function, which makes the development of a stable formulation difficult[19]. The application of this method to several different drugs has been reviewed[68].

An analogous thiazolium system[69] and carrier groups that involve alkoxycarbonyl methyl derivatives of 7,4-dihydropyridine-3,5-dicarboxylate[70] have also been proposed to overcome stability problems of the original prodrugs.

1.3. Prodrugs for amines

Section 1.3 will review the published attempts to produce prodrugs for amine drugs, as this is the principal focus of this thesis.

Amines pose several difficulties to drug developers due to their ionisation characteristics, their poor lipophilicity, which generally leads to poor membrane penetration, and their poor stability. First pass metabolism also leads to low bioavailability of primary amines due to N-acetylation and oxidation by monoamineoxidase (MAO)[71]. The same applies to peptides[72] containing basic amino acid side-chains. Low water solubility, poor stability and low permeability through biological membranes, often hinder the clinical development of biologically active peptides[73].

Although peptides can be derivatised on other functionalities, in this chapter only the approaches that involve the amino group alone or conjointly with other functional groups will be reviewed.

At the end of this section a table will be presented (page 39) as a summary of the more relevant approaches to the preparation of prodrugs for the amino group.

1.3.1. N-Alkylation

Some N-Alkylated derivatives of 2-phenylethylamine (PEA) were tested as substrates for monoaminoxidase (MAO). N,N-dipropargyl-2-phenylethylamine and N-propargyl-2-phenylethylamine were found to be inhibitors of MAO-B and to increase the brain levels of (PEA) in rats while significantly decreasing the whole brain concentrations of noradrenaline[74,75].

While administration of PEA only causes transient increases of PEA in the brain, N-(2-cyanoethyl)-2-phenylethylamine[76] and N-(3-chloropropyl)-2-phenylethylamine[77] caused sustained elevations of PEA.

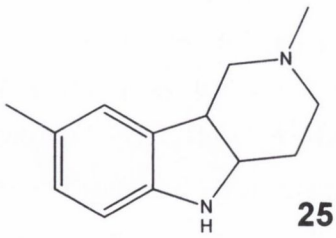
No other references to these types of prodrugs were found.

1.3.2. N-Acylation and carbamation

As seen before, bioreversible masking of hydroxyl or carbonyl groups by an ester functionality is a practical approach as these protected compounds can be cleaved in most biological systems by esterases. However, applying such a strategy to the preparation of prodrugs for amines is not always feasible, because of the relative chemical and enzymatic stability of amide bonds under physiological conditions, which makes the regeneration of the original drug more difficult. Amine derivatives of phthalamic acid, for example, despite being readily hydrolysed in acidic conditions due to intramolecular catalysis (releasing the amine and phthalic acid), are much more stable at pH=7.4 and in human plasma, rending them unsuitable as prodrugs[78].

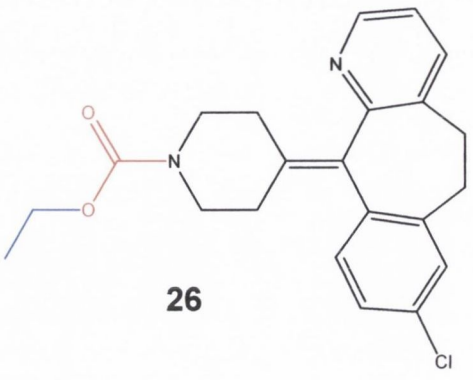
Therefore, simple N-acylation of amines to produce amides or carbamates is of limited use unless the compound produced is somehow activated by electron-withdrawing substituents or if it can be cleaved by particular enzymatic systems. One example that has been extensively studied is allopurinol whose N-acyl derivatives are more lipophilic than the parent drug while, and, at the same time, some of them are also more water soluble[79].

Recently the concept seems to have been subjected to a revival as several new examples have appeared in the literature. One such example is stobadine (**25**) whose acyl derivatives (acetyl, valeroyl and nicotinoyl) are highly lipophilic and penetrate the BBB[80].



25

Loratadine (**26**), a non sedating anti histamine, is another notable example as it was initially thought to be a non sedating antihistaminic drug and it was later found to be metabolised to desloratadine (**27**) which is actually the active drug[81].



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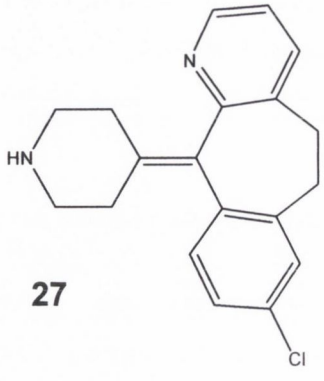
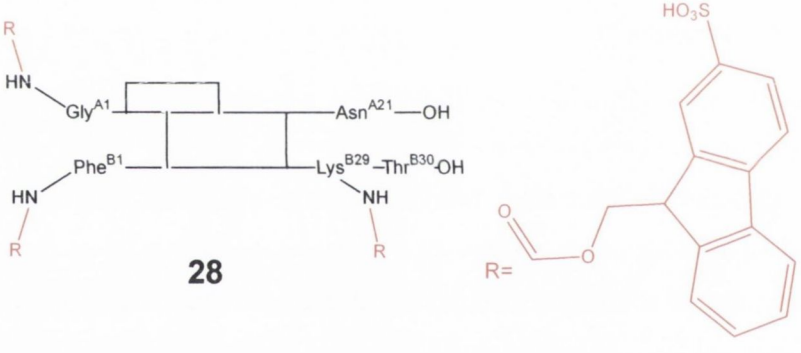


27

Another example, which relies on the slow cleavage for the preparation of long acting prodrugs, is that of [(2-sulfo)-9-fluorenylmethoxycarbonyl]$_3$ derivatives (**28**) of glucose lowering drugs like insulin[82] (**28** R=H) and exendin-4[83]. The prodrugs have the advantage of delivering the drugs slowly to the systemic circulation reducing the risk of hypoglycaemia.



28

Some N-acyl systems are cleaved by enzymes. As mentioned before (page 17), derivatisation of amines with γ-glutamic acid has received much attention due to the fact that the kidney has high concentration of γ-glutamyl transpeptidase. The enzyme breaks these acyl derivatives thus allowing the production of kidney specific prodrugs of amines[20].

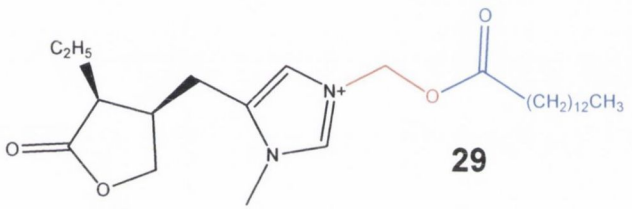
Some glycine and valine amides of monoaminooxidase A (MAO-A) inhibitors were also prepared as an attempt to deliver the active compounds to the brain before inhibiting MAO-A in the intestine mucosa which leads to the potentiation of tyramine induced hypertension[84]. More recently, amino acids have also been linked to anti tumour amines to produce water soluble amide prodrugs that release, *in vivo*, the original amine[85].

Another system that involves carbamate and urea prodrugs has been developed for antibody-directed enzyme prodrug therapy. In this system, the carbamate and the urea groups are substrates for the enzyme tyrosinase that can be delivered to melanomas where it triggers the release of the drug[86].

New cephalosporins have also been subjected to acyl derivatisation in order to increase solubility[87].

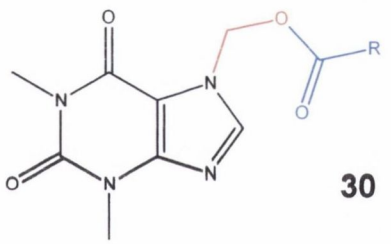
1.3.3. N-Acyloxyalkylation, N-hydroxyalkylation and (phosphoryloxy)alkylation

N-Acyloxyalkyl derivatives of primary and secondary amines are not usually suitable as prodrugs due to their high lability in aqueous solution but with tertiary or N-heterocyclic amines it is possible to produce stable quaternary ammonium salts susceptible to enzymatic hydrolysis and subsequent spontaneous decomposition as in the case of the tetradecyloxymethyl quaternary salt of pilocarpine[37] (**29**).



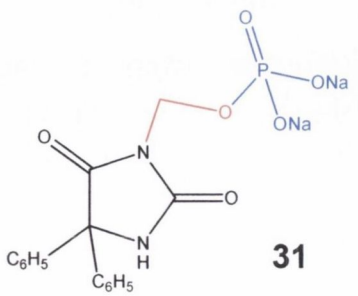
29

N-acyloxyalkylation was also tested on the topical antiproliferative drug theophylline but, no real improvement in the permeability characteristics through the skin were observed with alkyl moieties with up to five carbon chains (**30**). However, the intermediate 7-hydroxymethyltheophylline, delivered twice the amount of theophylline as the drug itself[88].



30

N-phosphoryloxyalkylation as also been applied namely to the anti-epileptic phenytoin (**31**) to produce forsphenytoin which is a notable example of a useful prodrug[89].



31

1.3.4. (Acyloxy)alkyl and (phosphoryloxy)alkyl carbamates

N-Acyloxyalkoxycarbonyl derivatives or (acyloxy)alkyl carbamates (Figure 1.9, R_4= alkyl or aryl), can be used as prodrugs for amines[7,90,91,92]. These compounds are enzymatically hydrolysed in vivo releasing the (hydroxyalkoxy)carbonyl derivative that spontaneously decomposes to the parent amine via a labile carbamic acid. Since the compounds are not ionisable at physiological pH, they are more lipid soluble than the parent amines[93].

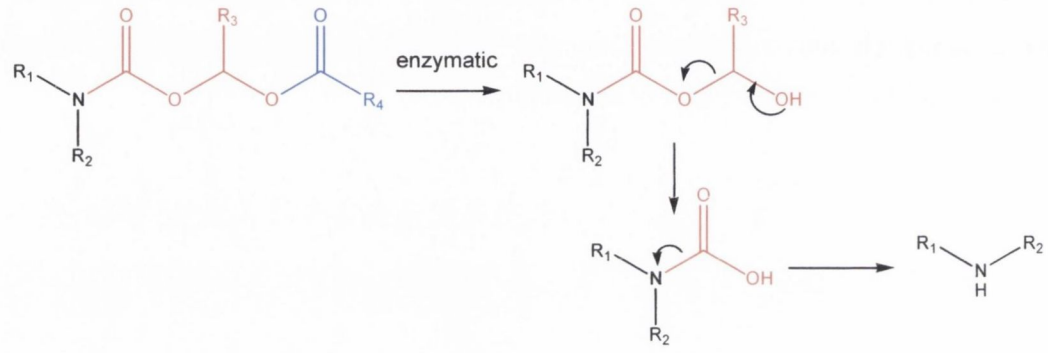


Figure 1.9: Hydrolysis of (acyloxy)alkyl carbamate prodrugs of amines

The system may however be of limited use for primary amines as an intermolecular acyl transfer reaction may occur, leading to the formation of a very stable N-acylated amine, reducing the yield of the regenerated amine[8].

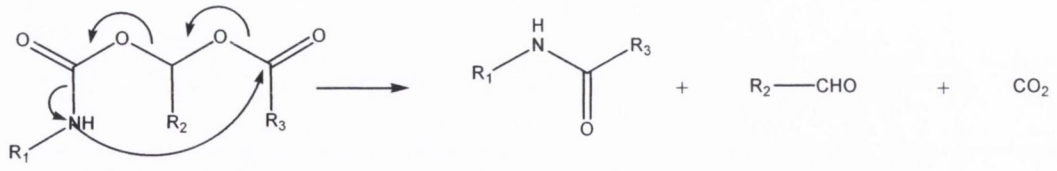
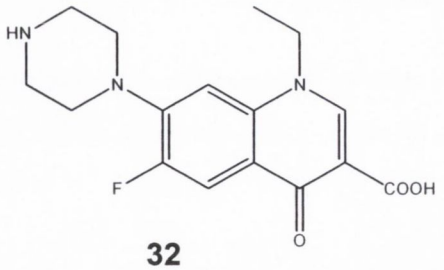


Figure 1.10: Intramolecular acyl transfer in N-acyloxyalkoxycarbonyl derivatives of primary amines

(Acyloxy)alkyl carbamates derivatives of hydrophilic beta-blockers demonstrated several fold increase in rat skin and rabbit cornea permeation by comparison with the original drugs[94].

Other examples of (acyloxy)alkyl carbamates are the tasteless prodrugs (**32a**) of the bitter anti-bacterial Norfloxacin (**32**)[95].



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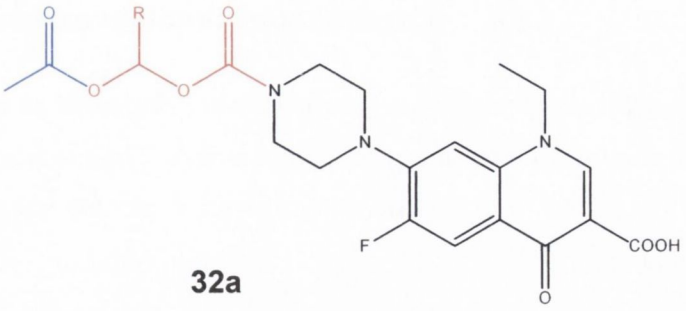
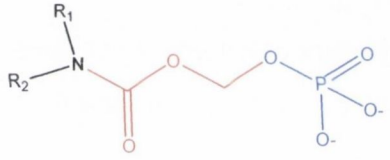


32a

The use of (acyloxy)methyl esters (Figure 1.9, R_3=H) is generally a topic of controversy due to the generation of formaldehyde during breakdown. For this reason, (acyloxy)ethyl esters (Figure 1.9, R_3=CH_3) are usually preferred. On the other hand, (acyloxy)ethyl derivatives introduce a chiral centre in the system; if the drug already has a chiral centre, diastereomers are formed and can display very different hydrolytic rates[93].

(Alkoxycarbonyloxy)methyl carbamates have also been prepared (Figure 1.9, R_4= alkoxy or aryloxy)[92] as well as (phosphoryloxy)methyl carbamates (**33**) which would, *in vivo*, be cleaved by alkaline phosphatases. *In vitro* tests showed that, following the initial enzymatic triggering, a spontaneous cascade leads to the release of the amine[96].



33

1.3.5. Quaternary derivatives of tertiary amines

Some quaternary derivatives of tertiary amines have been mentioned as potential prodrugs since these compounds degrade at physiological pH releasing the parent amine[20]. Examples are the already mentioned acyloxyalkyl derivatives (section 1.3.3). Another approach involves a salt form of a N-phosphonooxymethyl prodrug, from which the parent drug is released by a first step enzyme-catalysed rate-determining dephosphorylation, followed by spontaneous chemical breakdown of the N-hydroxymethyl intermediate (Figure 1.11)[97].

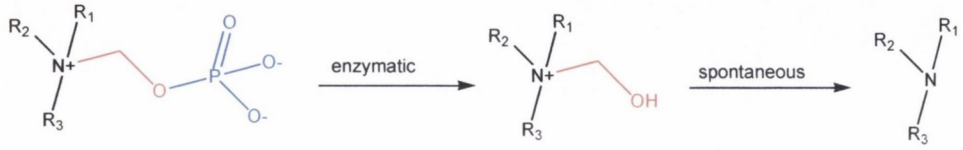


Figure 1.11: Tripartate prodrug system for tertiary amines

The approach improves the aqueous solubility and stability of the drug[98] and, *in vivo* tests, suggest that there is quantitative reversion of the prodrug to the parent drug[99].

1.3.6. (Oxodioxolenyl)methyl carbamates

(Oxodioxolenyl)methyl carbamates were prepared as an attempt to avoid the drawbacks of (acyloxy)ethyl and (acyloxy)methyl esters. The prodrugs break by base catalysis according to Figure 1.12 but the rate of hydrolysis in plasma solutions are higher than in pH 7.4 buffer solutions[92,93].

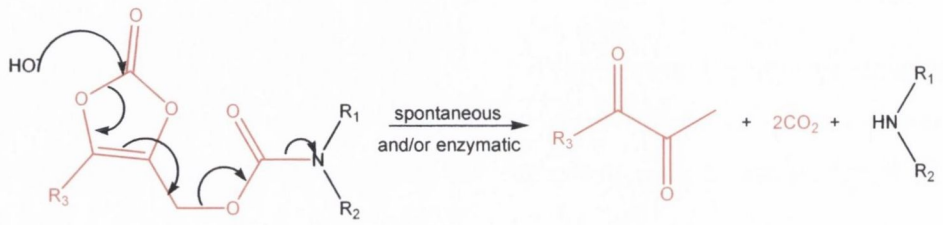


Figure 1.12: (Oxodioxolenyl)methyl carbamate prodrugs of amines: mechanism of base catalysed cleavage

In these systems, the cleavage of the dioxolenone ring by the amino group attack on the reactive vinylene carbonate function is precluded which makes this approach potentially applicable to primary amines as well. Aryl R_3 substituents generally have a destabilising influence reducing the half-life of the prodrugs[93].

The system has been applied to pseudomycins and some of the prodrugs exhibited comparable *in vivo* efficacy to that achieved by the parent compounds with reduced side effects[100].

1.3.7. N-Mannich bases

N-Mannich bases are synthesised through the Mannich reaction that involves a NH-acidic compound, an aldehyde (usually formaldehyde) and an amine (Figure 1.13) in ethanol.

This system can be used to produce prodrugs that are more soluble than the parent drug[101] not only for amines but also for amides as in the case of rolitetracycline[8].

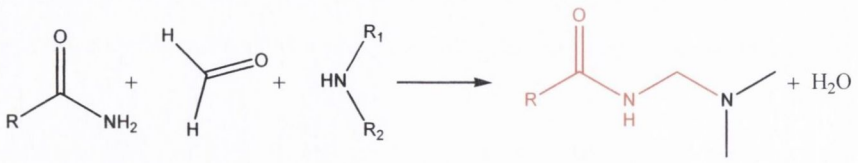


Figure 1.13: Synthesis of N-Mannich bases

N-Mannich bases are useful when an increase in the lipophilicity of amines is desirable. The new compound has a significantly lower pK_a (a difference of up to 4 units) in comparison to the original amine, which means that an important proportion remains

unionised at the pH of the intestine[102]. However, the selection of biologically acceptable, amide type, transport groups affording an appropriate cleavage rate, is restricted[26].

Cleavage of the prodrug, in this case, is strictly pH mediated and it has been found that N-Mannich bases of salicylamide and different aliphatic amines and aminoacids show a bell shaped pH/rate profile with high breakdown rate at pH 7.4. In the case of salicylamide, the hydroxyl group is thought to be responsible for the high reactivity, possibly by intramolecular catalysis, when the compound is in the neutral or zwitterionic forms. At high pH, when the compound is in the anionic form, the reactivity decreases markedly[102]. However, derivatisation of this group by acyloxymethylation, provides new possibilities of controlling *in vivo* cleavage as well as improved *in vitro* stability (Figure 1.14) [103].

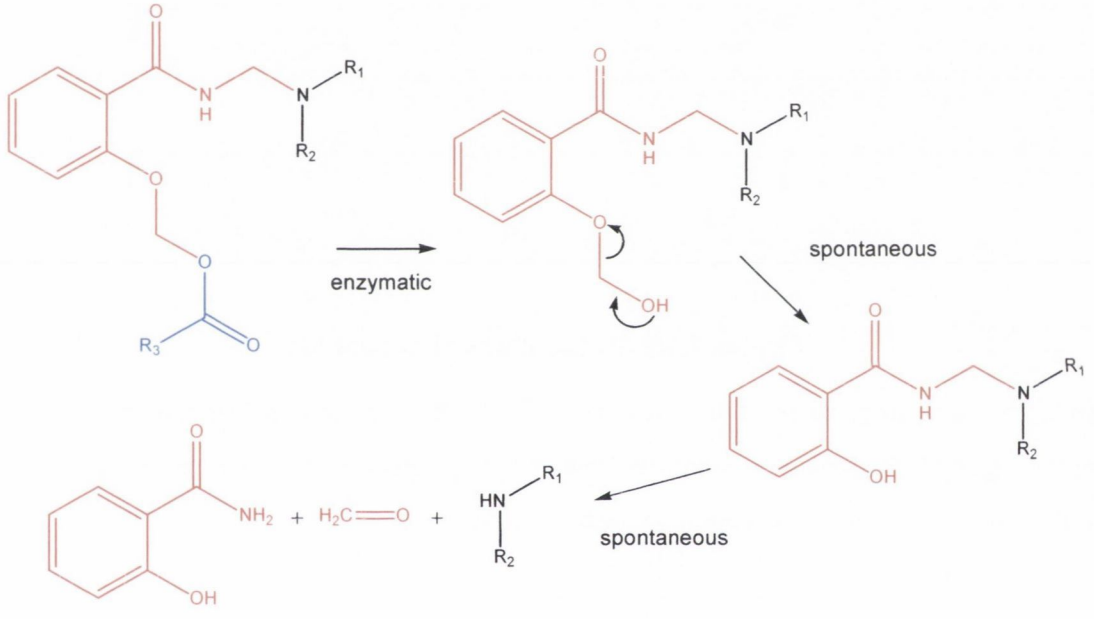


Figure 1.14: Esterase sensitive N-Mannich bases of salicylamide as prodrugs for amines

Studies on structure-reactivity relationships established that acidity of the parent amide and steric effects within the amine component can be correlated with reactivity[101,104]. For amines with similar steric properties, a decreasing basicity is associated with decreasing reactivity[101]. For example, the rate of breakdown of N-Mannich bases of aromatic amines with succinimide increases markedly with increasing amine basicity[71]. For amines with similar pK_as, some correlation was found between reaction rates and the difference in pK_a between the amine and the corresponding Mannich base (for the same amide)[105].

A drawback of the N-Mannich bases is their limited stability *in vitro*, raising some stability/formulation problems[8]. The unavoidable release of formaldehyde during decomposition is another factor that has to be taken into consideration due to its toxicity[101].

1.3.8. Schiff bases and azo compounds

Imines (Schiff bases), are usually too easily hydrolysed to be used as prodrugs for amines. Nevertheless, in some particular cases they may be surprisingly stable and useful as they impart increased lipophilicity to the parent amine and depress the pK_a value. The anticonvulsant progabide (**34**) was prepared as a prodrug form of γ-aminobutyric acid (GABA) since it crosses the BBB, while the free drug doesn't. Once in the brain, it is converted to γ-aminobutiramide and GABA. Nevertheless, it cannot be considered a true prodrug as it has some pharmacological effects by itself[20].

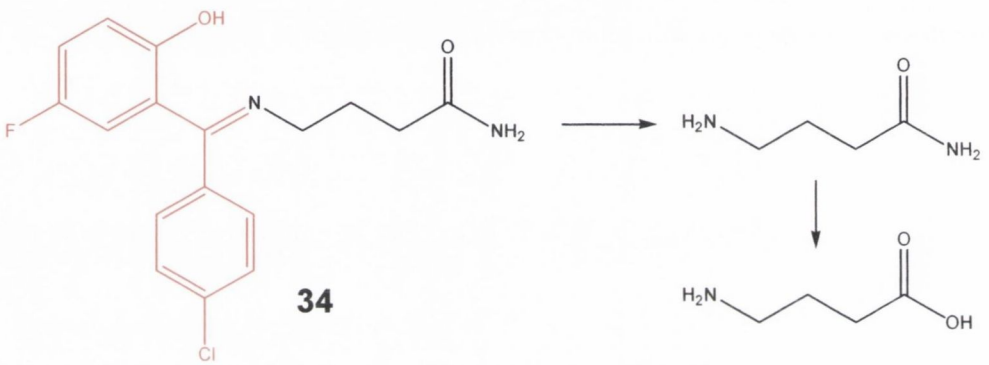
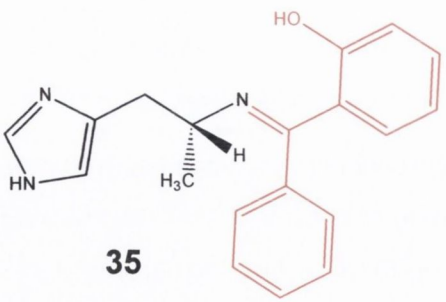


Figure 1.15: Metabolism of progabide

Chemically activated azomethine prodrugs[106,107] (**35**) of the reference histamine H3 receptor agonist *R*-α-methylhistamine have been prepared, as well as enzymatically[108] activated prodrugs (amide, esters and carbamates).



The new compounds, being more lipophilic, have improved oral absorption and better brain penetration than the original drug.

Azo compounds have been produced as site-specific prodrugs that exploit the facile reduction of the azo linkage by azo-reductase enzymes. One successful example of this is the 5-aminosalicylic acid prodrug, olsalazine (**23**), previously mentioned (page 17).

1.3.9. Enamines and enaminones

Enamines[109], (α,β-unsaturated amines) just like imines, are very unstable particularly at low pH, which make them unsuitable for the preparation of prodrugs for oral delivery. Nevertheless, an enamine prodrug of ampicillin was found to promote the rectal absorption of the drug[110].

Enaminones, which are enamines prepared from β-dicarbonyl compounds are more stable, probably due to intramolecular hydrogen bonding (Figure 1.16) and were thought to have potential use as prodrugs[20].

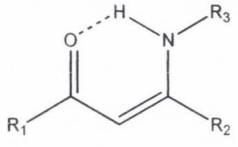


Figure 1.16: Intramolecular hydrogen bonding stabilisation of enaminones

The hydrolysis of enaminones derived from some aminoacids and antibiotics is fairly rapid, releasing the amine and a diketone.

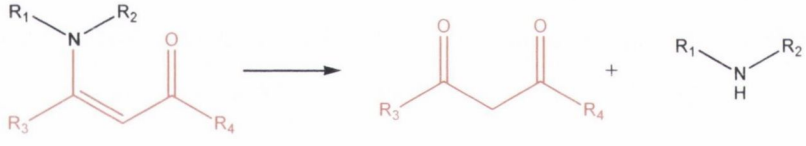


Figure 1.17: Hydrolysis of enaminones

The prodrugs are more lipophilic than the corresponding drugs, which usually results in improved absorption[20]. The system seems to be relatively insensitive to the type of amine used but very sensitive to minor changes in the structure of the 1,3-dicarbonyl compound used to produce the prodrug. Closed structures like the compounds derived from cyclohexane-1,3-dione show considerably lower rates of hydrolysis. This is probably due to their rigid geometry and the inherent stability of this system. The maximum rate of hydrolysis occurs in the pH range 2-5[111].

Based on chemical stability considerations, enaminones do not seem promising as prodrugs. However it has been speculated that enaminones obtained from ketoesters and lactones may be better candidates as they may be subjected to enzyme-catalysed degradation[112].

1.3.10. "Trimethyl lock" and coumarin systems

Phenolic amides derived from lactones can be used as amine prodrug systems as they release the amine and a lactone at the physiological pH[113] (Figure 1.18). In this system, referred to, generally, as a "trimethyl lock", the side chain is folded back to bring the side chain carboxyl group to the proximity of a phenolic oxygen that is the potential nucleophile. This conformation may account for the facile cyclisation that occurs independently of drug attached to the side chain[114]. However the half-lives of these systems are usually less than 1 min which is too short for a useful application[115].

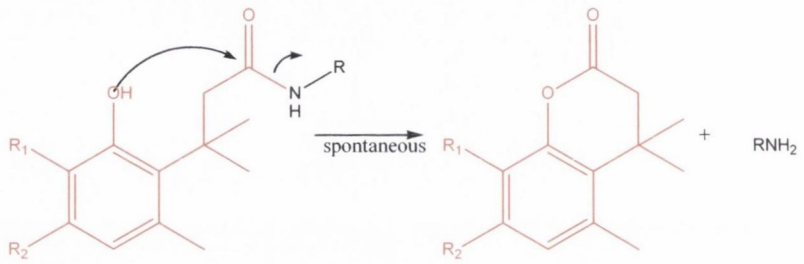
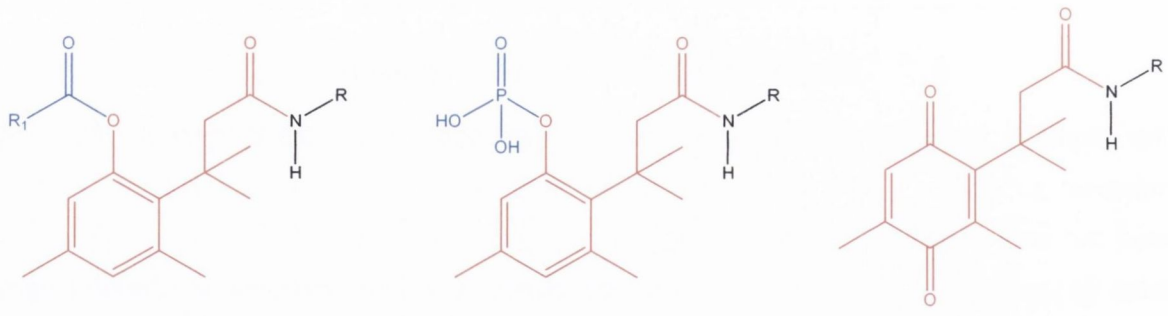


Figure 1.18: "Trimethyl lock" prodrug system for amino drugs

Nevertheless, the system can be improved by its transformation into a chemically stable compound that is enzymatically[115] or redox[116] sensitive (Figure 1.19). These derivatisations involve the protection of the nucleophilic hydroxyl in a bioreversible manner. The rate-limiting step then becomes the enzymatic exposure of the phenolic group.



Esterase sensitive | Alkaline phosphatase sensitive | redox sensitive

Figure 1.19: Tripartate "trimethyl lock" systems

A system combining hydroxy amide lactonization with phosphate esters as substrates for alkaline phosphatase, was also developed[117].

A similar system with somewhat better performance, takes advantage of the facile cyclization of coumarinic acid and its derivatives[118] (Figure 1.20). The presence of the phenolic hydroxyl group and the *cis*-geometry of the double bond allows lactonization at rates comparable to those of the "trimethyl lock" system. The phenol group is protected by

an ester or a phosphate group that serves as an esterase or phosphatase sensitive biological trigger.

The lactonization step seems to be determinant on the release rates that are generally higher for primary amines than for secondary amines; it also depends on steric features of the amine to be released[119]. For secondary amines with high pK_as, the system is sometimes undesirably slow. Nevertheless, different substituents on the aromatic ring, have significant effect and may increase the release of the drug by as much as 16-fold[120,121].

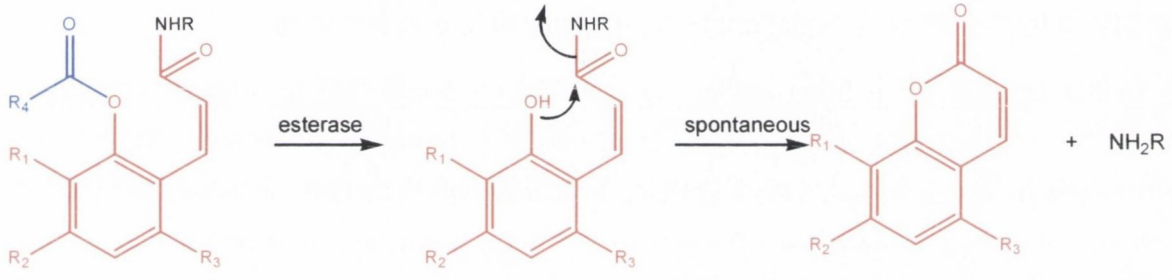


Figure 1.20: Coumarin-based esterase sensitive system for amino drugs

More recently, an attempt was made to prepare a tripartate prodrug (double prodrug) that uses the coumarin system as a spacer between the drug (linked to the side chain) and a carrier group, a peptide or an aminoacid, connected to the hydroxyl group of the coumarin. The advantage of this system would be the possibility of targeting drug specific proteases for the cleavage that would release the carrier, which would be followed by spontaneous lactonization, releasing the drug. Poor aqueous solubility has however limited the exploitation of this system[122].

1.3.11. Redox systems

Reductive elimination, which constitutes an example of a bioprecursor system rather than a carrier system, has received significant attention recently, mostly for amine drugs. Several reductive systems to be activated in hypoxic conditions, were developed and applied, particularly in cancer therapy. The systems involve amines latent as nitro groups that, after conversion, are able to form adducts with DNA. The reduction pathway involves several radical intermediates as well as nitroso, hydroxylamine and amine intermediates (Figure 1.21).

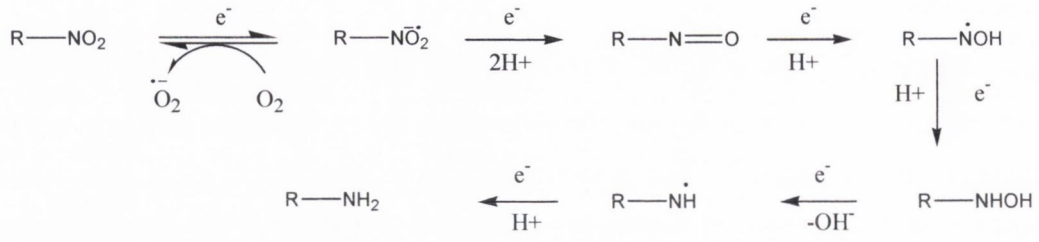
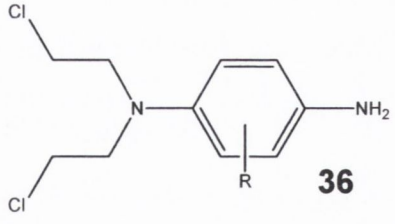


Figure 1.21: Generalised reduction pathway for nitro-heterocycles[123]

Several systems of this type have been used such as 2-nitroimidazoles, mitomycin C, tirapazamine[123] and indolequinones[124], amongst others. The reduction is normally facilitated by the action of endogenous or specially delivered enzymes.

A similar approach has been applied to aromatic mustards (**36**) to produce prodrugs to target hypoxic tumours. Although the direct use of nitroaromatic mustards (**37**) could be envisaged as a hypoxic activated system, its application is limited because both the nitro and the alkylating groups are attached to the same aromatic rings and have opposing electronic requirements. This means that the prodrug has a very low reduction potential with consequent low hypoxic selectivity. Substitution on the aromatic ring, with electron-withdrawing groups, in order to elevate the potential is possible but greatly reduces the cytotoxicity of the drug[125].



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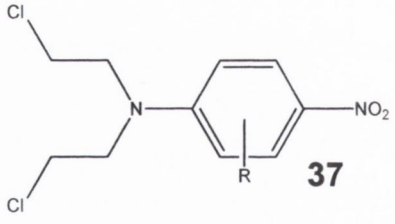


37

However, the design of a reductively activated system where the reduction potential can be manipulated independently (R_1) allows the production of prodrugs of the more cytotoxic non-substituted mustards. Moreover, the selection of the linker group X (Figure 1.22) allows some control over the rates of cyclization[125].

A series of N-dinitrophenylamino acid amides, which also release primary amines via nitroreduction and intramolecular cyclization, has been studied. This system does not seem to be efficiently activated by nitroreductases but the reduction can be radiation-induced, which is a possible approach in cancer therapy[126].

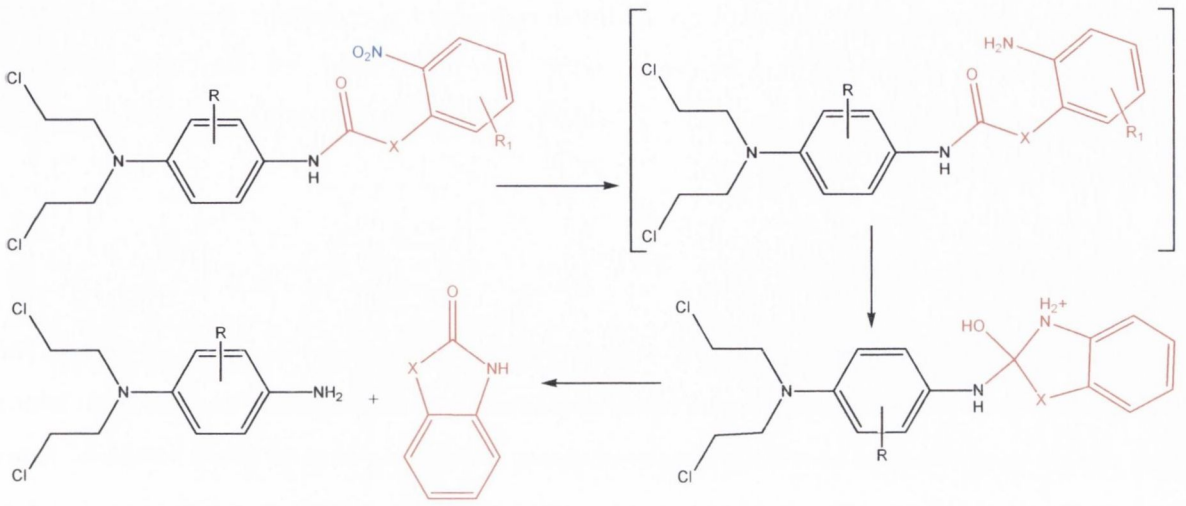


Figure 1.22: Mechanism of activation of 2-aminoaryl derivatives

Another system that can be activated by reduction is the 4-azidobenzyloxycarbonyl (Figure 1.23). In this system the drug is linked through a carbamate to an aromatic azide that is converted by reduction to an amine. After that a cascade reaction eliminates the carbamic acid, which is readily converted to the amine drug[127].

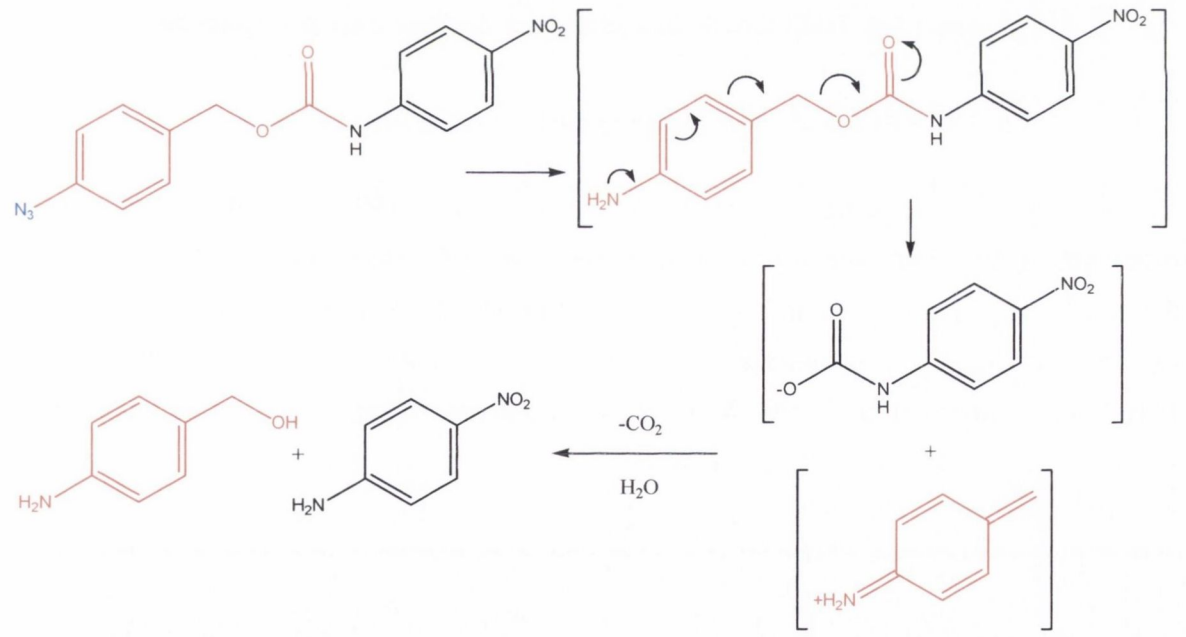


Figure 1.23: Activation of the 4-azidobenzyloxycarbonyl prodrug system

N-oxides have been suggested as bioreductive prodrugs for tertiary amines. N-oxidation masks the cationic charge of the amine reducing their DNA binding affinity and their toxicity. The prodrugs are activated by metabolic reduction under hypoxic conditions[128].

The aforementioned dihydropyridine system (page 21) is another example of a site-specific prodrug, developed for brain penetration of amines (but that is also applicable to alcohols

and carboxylic acids), which involves an oxidative pathway for activation. Application of this system in amines was illustrated for desipramine, amongst others. In this case, although there was no evidence of a more efficient delivery, there was a prolonged presence of the drug in the rat brain at a constant level[129].

1.3.12. THTT

A tetrahydrothiadiazine-2-thione (THTT, Figure 1.24) was proposed as prodrug system for primary amines[130], amino acid[131] and peptide drugs[132]. In this system the nitrogen atom from the drug is included in a six membered cyclic compound that is more lipophilic than the original drug. The prodrugs are enzymatic and chemically sensitive at the physiological pH but stable under acidic conditions. Despite the apparent promise of this system, it does not seem to have been subjected to further developments

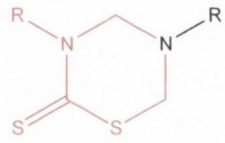


Figure 1.24: THTT prodrug system for amines and aminoacids

1.3.13. PEG and other macromolecular systems

Double prodrug systems that consist of poly(ethylene glycol) (PEG) linked through a spacer to the amine drug (**38**), proved to be advantageous to facilitate solubilization of insoluble drugs. In one approach, ester, carbonate, carbamate or amide bonds are introduced as spacers and triggers for enzymatic activation and release of the PEG group. After that, the drug, that is latentiated in the form of a carbamate or an ester, is released by a spontaneous 1,4- or 1,6-benzyl elimination[133,134].

38

PEG can also be used in conjunction with the trimethyl lock system described above to produce prodrugs for amines with improved solubility and potentially capable of targeting specific tumours. In this system, PEG is connected through a spacer to the phenol group of the open lactone. Manipulation of the spacer or the substituents in the aromatic ring allows for manipulation of release rates[134,135,136,137].

Low molecular weight proteins have also been used, mainly to prepare systems for kidney targeting that can be cleaved by aminopeptidases or lysosomal lysates. The LMWP can also be linked to the drug through an acid sensitive spacer. Using β-naphtylamine (β-naph) as a model compound, the Leu-β-naph and the Gly-Phen-β-naph conjugates were stable in buffer solution but released the amine completely in cortex homogenates and lysosomal lysates solutions. However the results were not as promising with adriamycin, triametrene and sulfamethoxasole as model drugs[51].

1.3.14. Cyclic derivatives of polyfunctional drugs containing the amino group

Making prodrugs of compounds with multifunctional groups may be a challenge, particularly in the case of peptides where the amine group poses a problem of its own due to the lack of suitable methods of biologically reversible derivatisation. The "trimethyl lock" and coumarin systems mentioned before as prodrug systems for amines, have also been tested as prodrugs for peptides (linked to the progroup through an amide and an ester link) with promising results[138,139,140,141].

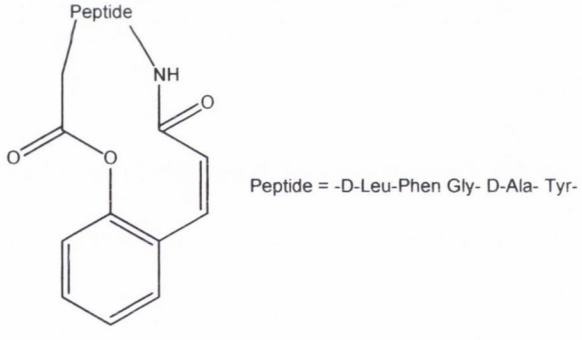
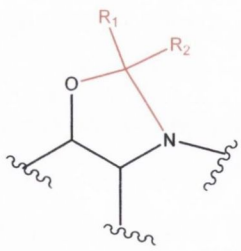


Figure 1.25: Coumarin system applied to peptides

The concept has been applied to peptides like DADLE, an opioid peptide. The C and N terminal ends of the linear peptide are masked by forming an ester and an amide bond with the phenol hydroxyl and side chain carboxyl groups, respectively, of the linker[138].

Oxazolidines (**39**), which are cyclic condensation products of β-aminoalcohols and aldehydes or ketones, are a possible means of producing prodrugs for these functional groups[26]. These compounds are less basic and more lipophilic than the corresponding β-aminoalcohols and hydrolyse completely in aqueous solution[142]. Thiazolidines can be used for β-aminothiols.



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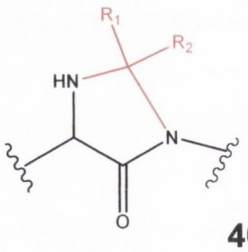

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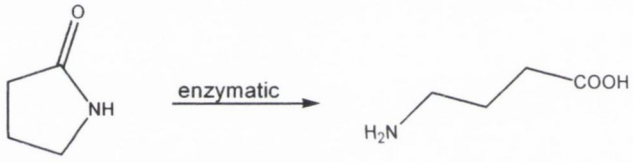
4-Imidazolidinones[143,144] (**40**) have been proposed for the α-aminoamide moiety, in particular as prodrugs of Leu-enkephalin and prilocaine. The derivatives of Leu-enkephalin afford protection against aminopeptidase N and angiotensin converting enzyme (ACE) and are cleaved slowly in buffered solutions at pH=7.4 with half-lives of some hundred minutes[145]. The hydrolysis of some prilocaine derivatives, at basic pHs, proceeds to an equilibrium due to reversible kinetics[144].

Lactams and pyrrolines have been shown to revert to the corresponding aminoacids by enzymic action. Moreover, they pass the BBB while the opened structures don't. One example is the cyclic derivative (**41**) of GABA depicted in Figure 1.26.

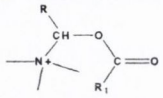
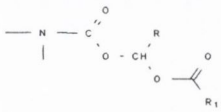
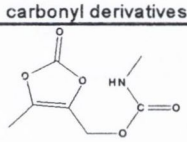
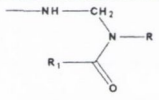
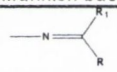
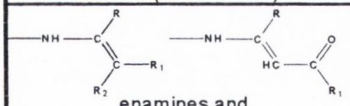
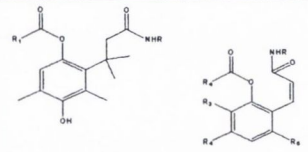
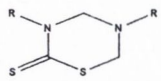
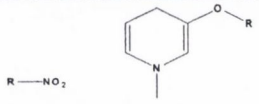


41

Figure 1.26: Conversion of 2-pyrrolidinone to GABA

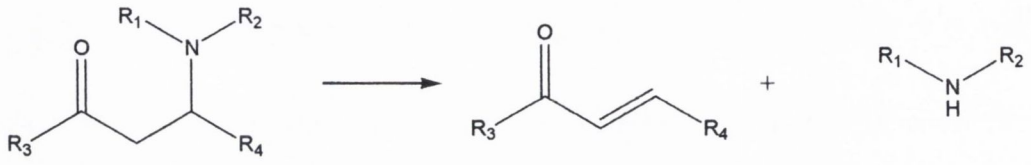
Table 1.1 summarises the most important types of prodrug systems that have been investigated for amines.

Table 1.1: Compilation of a series of published prodrug approaches to amine drugs

Prodrug compound	mechanism	advantages	disadvantages
 amines and carbamates	enzymatic, pH activated	lipid solubility may be improved slow release	Needs to be activated by an electron withrawing substitute
 N-acyloxyakylderivatives	enzymatic followed by spontaneous	improved lipophilicity	only applicable to tertiary amines
 N-acyloxyalkoxy carbonyl derivatives	enzymatic followed by spontaneous	produces neutral compounds	usually not suitable for primary amines
 (oxodioxolenyl)methyl derivatives	base catalysis and/or enzymatic	also applicable to primary amines	
 N-Mannich bases	base catalysis	lowers pKa up to 3 units	formation of formaldehide low stability
 imines (Schiff bases)	pH activated	lowers pKa	easily hydrolysed in aqueous solution
 enamines and enaminones	chemical	lowers pKa improved lipophilicity	not stable enough at low pH
azo compounds	azo-reductases	possibility of targeting	only applicable to aromatic amines
 lactonization systems	enzymatic followed by spontaneous	possible to manipulate phys/chem characteristics	poor aqueous solubility in most cases
 THTT	enzymatic and chemical	improved lipophilicity	only applicable to primary amines
 redox sytems	chemical or enzymatic activation	possibility of targeting	oxidation in solid state
PEG	chemical and enzymatic activation	improved solubility	need association with other systems

1.4. Origin and scope

In previous work done at our school, a series of β-aminoketones and β-amidoketones derived from 1-indanone demonstrated smooth muscle relaxing activity, mast cell stabilisation and anti-inflammatory activity[146]. While developing methods for their analysis and stability tests, it was observed that these β-aminoketones degraded in aqueous solution at rates that seemed to be correlated with the activity of the compounds.



Meanwhile we found that the compounds were stable in acidic conditions and the rates of degradation increased with increasing pH, releasing the original amine. Chapter 2 describes the work that lead to this discovery, including the development of chiral methods of analysis for separation and identification of isomers and metabolism tests.

As a reference to the reader, Annex 1 presents some information related to capillary electrophoresis that may be needed for a fuller understanding of the method choices.

The observations related to the degradation of the compounds have raised the idea of using this kind of system as prodrugs for amines. It was the aim of the work described in this thesis, to test this theory and prove its validity. With that purpose, new derivatives of 1-indanone were synthesised from model amines and drugs with the amine functionality. The pH/rate profiles of degradation of these compounds were evaluated by capillary electrophoresis and/or HPLC. The effect of temperature and ionic strength was studied. The pK_a values of the resultant compounds were determined to evaluate if there was any relationship between the rates of degradation and this parameter. Tests of absorption in the everted rat gut were done to investigate improvement in absorption imparted by inclusion of the promoiety. This work is described in Chapter 3

Chapter 4 presents work done with respect to β-amino ketones derived from carbonyl groups, others than 1-indanone, to test the generality of the prodrug system.

Some of these systems were applied to dopamine and L-dopa in an attempt to find prodrugs for Parkinson's or cardiovascular diseases. Chapter 5 describes these investigations.

Conclusion remarks and future work are presented in Chapter 6.

The experimental part of this work is described in Chapter 7.

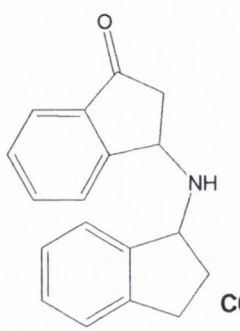
To distinguish the compounds prepared for this thesis from the other compounds referenced, a different numbering was used (C## and B## for target compounds and intermediates or degradation products respectively). Annex 2 presents a list of all these compounds as a quick reference for the reader.

CHAPTER 2. EARLY WORK ON β-AMINOINDANONES

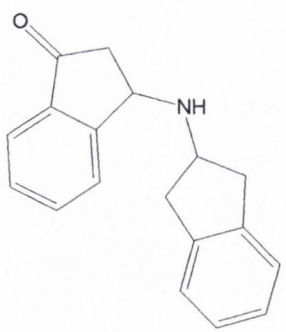
2.1. Introduction

The early objective of this project was to develop analytical methods for certain anti-inflammatory/antiallergenic amines, which had been discovered in the School of Pharmacy[146,147,148]. These methods were to be applied by the author to profiling the *in vitro* metabolism and bioavailability of these promising compounds.

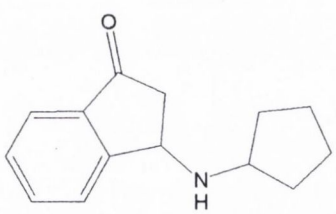
The compounds were β-aminoketones derived from primary (**C01**, **C02** and **C03**) or secondary (**C04**, **C05** and **C06**) amines and 1-indanone.



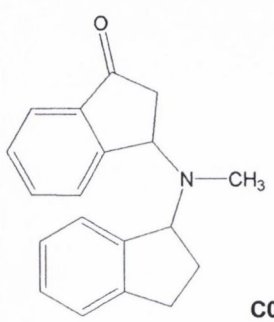
C01



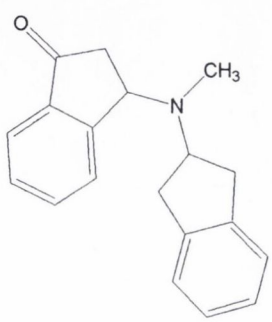
C02



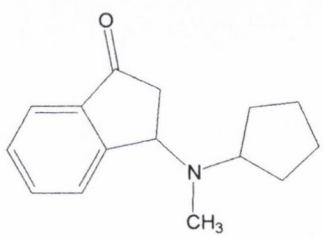
C03



C04

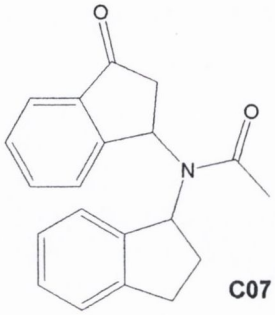


C05

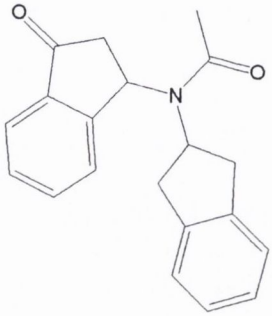


C06

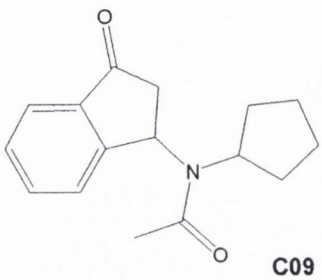
There were also amides (**C07**, **C08** and **C09**) prepared by acylation of the three original β-aminoketones.



C07

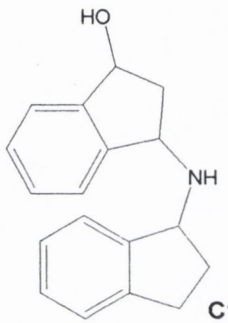


C08

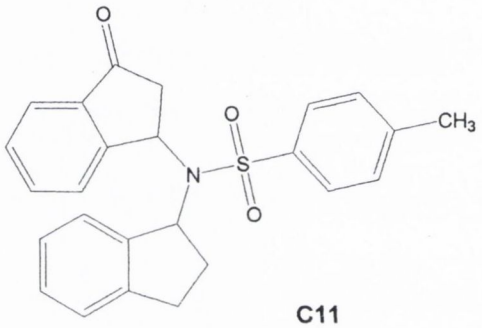


C09

The β-aminoalcohol (**C10**, obtained by reduction of **C01**) and the sulfonamide (**C11**) analogues of **C01** were also in the group.



C10



C11

This chapter describes the development of methods of analysis for these compounds and some P450 metabolism tests.

During this work, it was noted that some of the compounds were unstable in aqueous solution and studies of the kinetics of degradation were initiated.

2.2. Synthesis of test compounds

Although most of the compounds were originally provided by the research group that had tested them for pharmacological activity, new batches of some of the β-aminoketones had to be prepared. No new batches of compounds **C03**, **C06**, **C08**, **C07**, **C09**, **C10** and **C11** were prepared.

Confirmation of the identification of the compounds was made by NMR.

In the case of the β-aminoketones derivatives of indanone, the amine is attached at a benzylic position. Because of this, the strategy of synthesis was to perform a benzylic halogenation followed by the nucleophilic substitution of the halogen by the amine. N-bromosuccinimide (NBS) is the most common reagent used for this purpose and was the one chosen for this procedure, which is called *Wohl-Ziegler* bromination[149].

This type of reaction is usually performed in a non-polar solvent like carbon tetrachloride (where both NBS and the product succinimide are insoluble) and involves a radical mechanism. As initiators, light or peroxides are normally used (Figure 2.1).

Some bromination also occurred in the α position relative to the ketone and the two compounds were separated by flash chromatography.

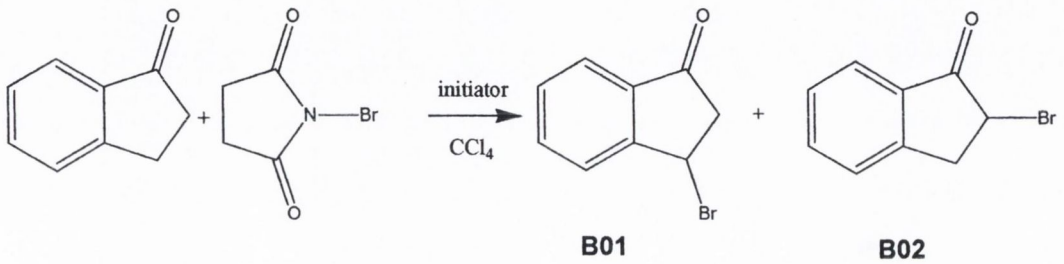


Figure 2.1: Wohl-Ziegler bromination (applied to indanone)

The following step consisted of the replacement of the bromine in the β brominated compound by the amine, which was performed in dry DCM in the presence of triethylamine (Figure 2.2).

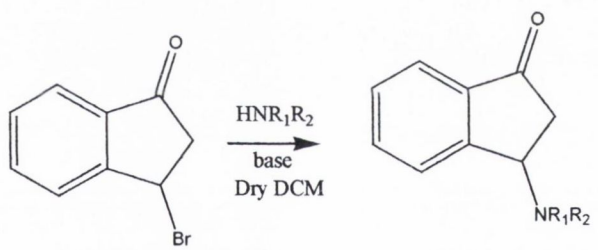


Figure 2.2: Nucleophilic substitution of bromine in 3-bromoindanone by an amine

N-methylation, to afford the tertiary amines (Figure 2.3), was performed with methyl iodide[146,148]. The compounds were purified by flash chromatography as described in Chapter 7.

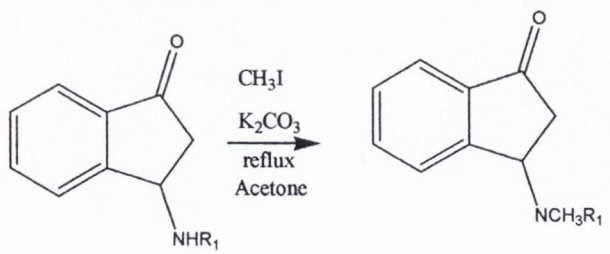


Figure 2.3: Synthesis of N-methylamines from secondary amines

The individual optical isomers of **C01** were prepared by using the optically pure forms of 1-aminoindane, thus obtaining two pairs of diastereomers, which could afterwards be separated by flash chromatography (Figure 2.4).

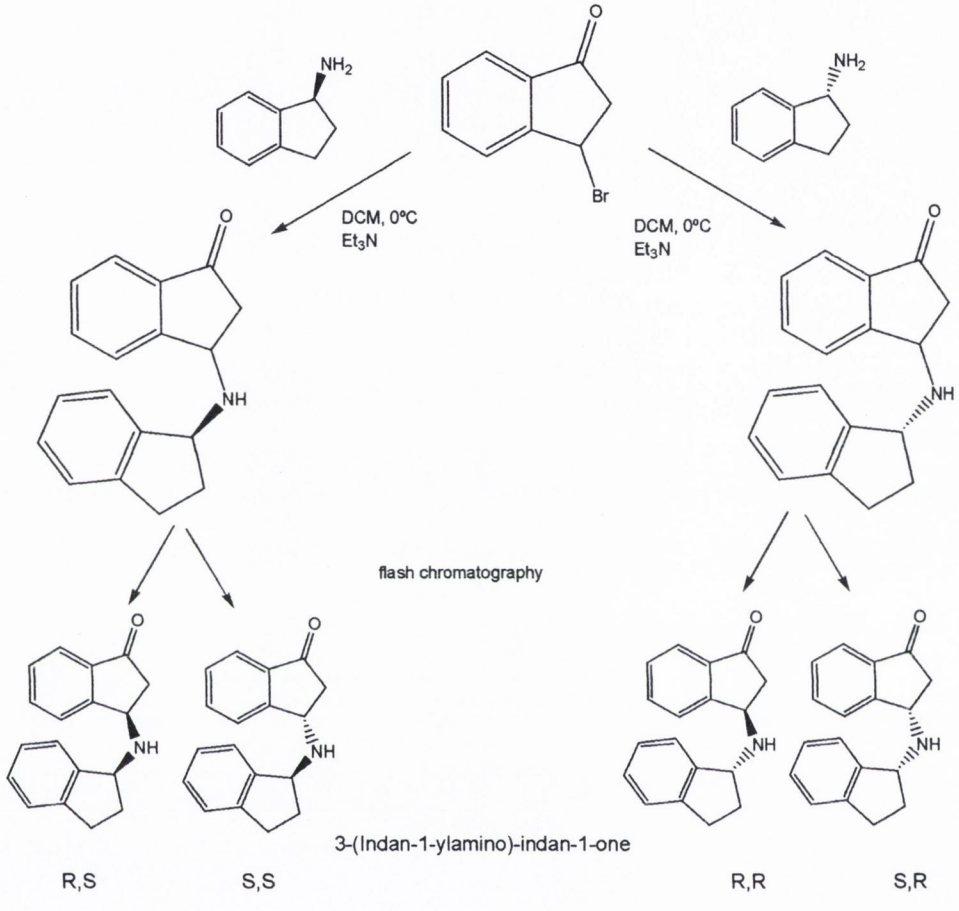


Figure 2.4: Synthesis and separation of the optical isomers of C01

2.3. Development of methods of analysis

HPLC and capillary electrophoresis methods were developed for the separation and analysis of the test compounds.

C01 was used as a model. The best methods developed for this compound were applied on the others, initially.

Capillary electrophoresis is still considered, by some, a recent technique. Since it is not as widely used as HPLC, some basic concepts and definitions are given in Annex 1 to help the reader understand some of the choices made in relation to method development.

2.3.1. Capillary electrophoresis methods

2.3.1.1. Non chiral running buffers and first tests with β-CD

For the initial tests, a 50 μm internal diameter silica capillary, 67 cm total length and 50 cm effective length was used. The temperature was 25ºC and a constant potential of 20 kV was used. Detection was originally made at 214 nm but, due to equipment constraints, more than methodological reasons, 200 nm was later preferred. Pressure injection was applied for 5 seconds.

During the course of the work it was observed that, once a method is set, the size of the capillary does not have great influence on resolution, as long as the applied potential or current is changed accordingly. As a general rule, voltages that produce currents between 50 and 150 μA, afford the best results. Smaller capillaries have the advantage of leading to smaller running times which is in fact one of the greatest benefits of capillary electrophoresis when compared to HPLC. Therefore, smaller capillaries were preferred towards the end of this work.

For this reason, in the description of the attempted settings for separation, the size of the capillary and the applied potential are not usually mentioned. However, Table 2.1, which shows the best CE methods found for each compound, at the end of this section, indicates an adequate potential and capillary size to work with each particular running buffer.

Two running buffers were originally tested at low and high pH to ascertain mobility and detectability of the compounds under these general conditions. **C01** was initially used as a model compound for each method. Improvements were attempted to allow better detection or resolution of the optical isomers and for analysis of the other compounds.

Normally, direct polarity (injection at the anode end of the capillary) was used. However, when using anions as selectors at low pH, injection polarity had to be reversed or injection had to be made on the end of the capillary close to the detector. Whenever this option was used, it is mentioned in the text.

With an acidic phosphate running buffer, a single peak was observed upon injection of aqueous solutions of **C01**, with migration times ranging between 7 and 12.5 min depending on the composition of the buffer. Phosphate buffers (10-100 mM) at pH=2.5 or 3 did not resolve the diastereomers of **C01** as expected since charge density should be the same for both, at this pH.

The addition of urea (3 M), which is a viscosity modifier, and β-cyclodextrin (β-CD) (80 mM) in conjunction, provided incomplete separation of four peaks with the following resolution factors: $Rs_{1,2}$=3.5, $Rs_{2,3}$=0.75, $Rs_{3,4}$=2.1. The addition of methanol increased retention times but did not improve resolution.

Micellar electrokinetic chromatography was attempted with basic buffers containing sodium dodecyl sulfate (SDS). A 10 mM Na_2HPO_4, 2.5 mM borax buffer (pH=9.5) containing methanol (33% v/v) and β-CD (7.5 mM) separated only two peaks of **C01** at 36 and 45 min respectively from a sample freshly prepared in water. Samples prepared in the running buffer originated more peaks.

2.3.1.2. Reversal of the EOF in acidic buffers

Since the electroosmotic flow (EOF) is inversely proportional to the ionic strength, it can be reduced by increasing the concentration of the buffer[150]. However, sometimes, this may not be feasible as an increase in the concentration of the buffer, also produces an increase in the current with consequent Joule heating and detrimental effect in peak shape.

Other possibilities include the use of modifiers of the dielectric constant of the running buffer like organic buffers or viscosity modifiers like urea.

It is also possible to use modifiers that interact with the capillary walls and alter the zeta potential. Such compounds can be for example tetraalkylammonium salts, which have the ability of completely reversing the EOF even in basic buffers[151].

Tetrabutylammonium phosphate salt (TBA) was used in the present work to reduce/reverse the EOF of both non-chiral and chiral buffers. The addition of this modifier (150 mM) to a phosphate buffer (50 mM, pH=2.5) produced a sharper peak for **C01** with longer migration time.

Later it was found that a 100 mM phosphate buffer at pH=3, containing 100 mM of TBA, provided a more robust and more repeatable method and these were the conditions chosen for quantitative non chiral analysis by EC.

Upon addition of 150 mM of TBA to a 50 mM phosphate buffer pH=2.5, in the presence of 20 mM of β-CD, three peaks were separated, one of them being about twice the area of each of the other two (Figure 2.5). Varying the concentration of β-CD between 15 and 95 mM did not improve separation.

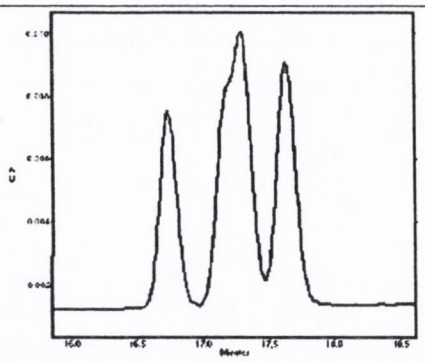


Figure 2.5: Partial separation of the optical isomers of C01 in the presence of β-CD and TBA

2.3.1.3. Manipulation of sensitivity by pressure or electrokinetic injection

Detection limit in CE is usually worse than in HPLC due to the very short light path of the capillaries. However, there are several procedures, which can concentrate (stack) the sample at the entrance of the capillary before separation, providing for peak sharpening and higher sensitivity[152].

These methods usually involve the use of discontinuous running buffers as in isotachophoresis or, more simply, the use of buffers with different compositions as the sample solution and for separation.

All these procedures are called *stacking* and they allow for higher injection volumes with consequent improvement on sensitivity, but without the effect of peak broadening usually associated with these large volumes. Due to the difference in conductivity, the sample components are focused at the boundary between the sample solution and the running buffer affording sharp peaks[153].

In the most common procedure the samples are dissolved in water or in low concentration buffers. High salt content solutions can also be used as well as organic solvents or molecules that interact with the analytes (like surfactants)[154]. Discontinuities in pH have also been used[152].

Most of these techniques can be applied either with hydrodynamic or electrokinetic injection. Certain CE manoeuvres, like the reversal of the applied polarity following injection can result in further improvements in the performance[155].

Pressure injection for 5 s, from a sample of **C01** in water, affords peaks with about the same area as the ones obtained by electrokinetic injection (5 kV) for the same period.

However, a 10 kV electrokinetic injection from aqueous solution produces peaks about three times the area of the peaks obtained with pressure injection for the same period. This increase is not observed if the test compound is dissolved in the running buffer. This may be due to the stacking effect occurring when the ionic strength difference between the running buffer and the sample solution is significant.

With high concentration saline solutions it was possible to increase injection times, and consequently the amount of sample injected, without significant peak broadening because the sample is focused at the entrance of the capillary. A three fold increase in sensitivity was obtained from a 60 mM sodium chloride solution by electrokinetic injection of **C01**, when compared to an aqueous solution: a further increase in salt concentration had a detrimental effect in the shape of the peaks.

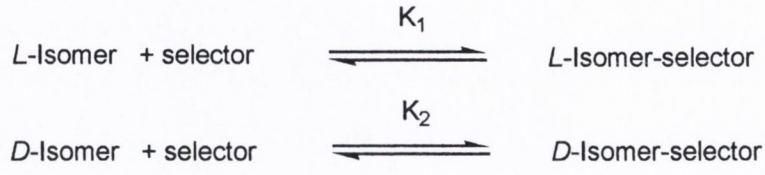
However these effects have to be taken into account when developing quantitative methods as a matrix effect can be significant. Since it is not always possible to prepare standards with the same characteristics as the matrix, a low time (3 s) pressure injection was preferred, whenever sensitivity was not an issue, because it provided for more repeatable results in different matrices.

2.3.1.4. Test of different chiral selectors

Chiral selectors and chiral compounds form transient diastereomeric complexes with different stabilities, which result in different solute migration and chiral recognition.

In order to achieve chiral separation, the molecule has to interact with the chiral selector in at least three positions. Ionic, hydrogen bond, π-π are some of the possible interactions.

The equilibrium constants between a chiral selector and each optical isomer have to be different in order to achieve separation. In this situation, the amount of time that each isomer spends linked to the selector is different and the migration times are affected accordingly.



If two isomers are not separated by a chiral selector, it does not necessarily mean that they are not interacting with the selector, but only that the interaction does not involve the chiral centre and consequently, $K_1=K_2$. A similar delay or advance in the migration time will be

observed for the two isomers in comparison to the migration times observed without the presence of the selector.

Sometimes MECC is used for chiral separations, either by the use of a chiral surfactant or by the association of a non-chiral surfactant with a chiral selector for example a cyclodextrin (CD-MEKC). In this case a secondary equilibrium is involved. The presence of organic modifiers in the running buffer may also affect the enantiomeric separation by altering the equilibrium partitions.

Several compounds have been used as chiral selectors, such as metal complexes, cyclodextrins (CDs), poly-peptides, proteins and biopolymers (affinity chiral selectors), bile salts (chiral surfactants), crown ethers, macrocyclic antibiotics and linear polysaccharides. Nevertheless, native and derivatised cyclodextrins continue to be the most widely used chiral selectors[156,157,158].

CDs are cyclic oligosaccharides consisting of 1,4-linked glucose units, either six (α-CD), seven (β-CD, Figure 2.6[159]) or eight (γ-CD)[160]. Some are derivatised (at the hydroxyl group) with charged or non-charged groups for further versatility. Selectivity by CDs is due to different equilibrium constants for the formation of inclusion complexes between the CD and each stereoisomer.

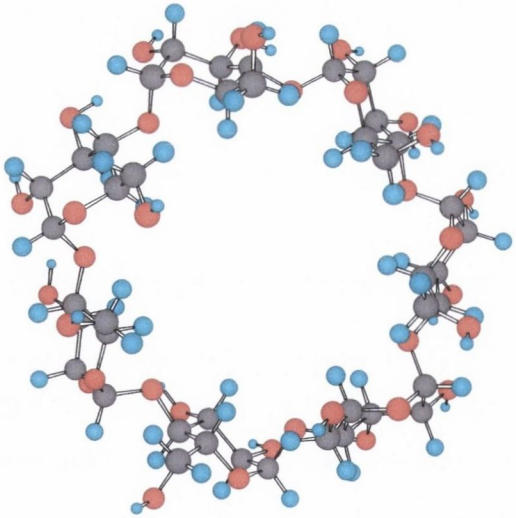


Figure 2.6: Structure of β-cyclodextrin (gray=C, red=O, blue=H)

In the present work, resolution of the compounds was attempted with different neutral cyclodextrins (CDs) at pH=2.5 (phosphate, 50 mM) and in the presence of TBA (150 mM). In a situation where direct polarity is applied, positive ions (like the amines), which interact with neutral CDs, are retarded because the weight of the adduct amine-CD is larger while the charge remains the same. Neutral compounds like the amides cannot be resolved by neutral CDs alone because the adduct amide-CD is neutral too.

γ-Cyclodextrin (γ-CD) and trimethyl-β-cyclodextrin (TM-β-CD) did not provide resolution of any compound at the tested concentrations (15 and 30 mM). α-Cyclodextrin (α-CD) afforded partial separation of **C01** and **C10** but not **C02** while methyl-β-cyclodextrin (M-β-CD) produced adjoining peaks from **C01**, **C10** and **C02**. **C03** only afforded one peak with all these systems. Mixtures of these CDs did not yield complete separation in any case.

Small concentrations of hydroxypropyl-β-cyclodextrin (HP-β-CD) afforded a slightly better separation (when compared to β-CD) of the previously observed three peaks of **C01**, but the use of dimethyl-β-cyclodextrin (DM-β-CD) leads to the complete separation of two peaks, each of them partially separated into another two.

However, concentrations of HP-β-CD of about 50 mM (dependent on the commercial source of the selector and probably, the degree of substitution, (Figure 2.7)) may or may not completely separate the four peaks. This difference in the resolution capacity of CDs from different sources had also been observed with β-CD.

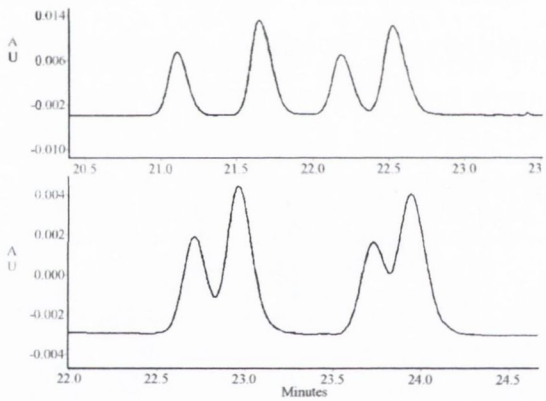


Figure 2.7: Difference in the resolution capacity of HP-β-CD from different sources

Apparently, β-CD and HP-β-CD (at low concentrations) are able to separate each pair of enantiomers but not the diastereomers. In contrast, DM-β-CD is able to separate the diastereomers but is not very efficient for the resolution of the enantiomers.

For this reason, mixtures of the different CDs were tested and good resolution was obtained with 15 mM of HP-β-CD and 15 mM of DM-β-CD in phosphate buffer (pH=2.5, 50 mM) and in the presence of TBA (150 mM). The migration times were between 20 and 25 min and the resolution factors were $Rs_{1,2}$=0.96, $Rs_{2,3}$=1.0, $Rs_{3,4}$=1.2. The order of migration of isomers in this running buffer is however different from the order of migration obtained with buffers containing only HP-β-CD at high concentration.

Organic modifiers like acetonitrile or methanol reduce the migration times but have detrimental effect on separation.

Reduction on the capillary size has a dramatic effect on the migration time but only slightly affects resolution (Figure 2.8, 20 cm capillary, 100 μA, 15 mM DM-β-CD, 15 mM HP-β-CD, 150 mM TBA in 50 mM phosphate buffer pH =2.5).

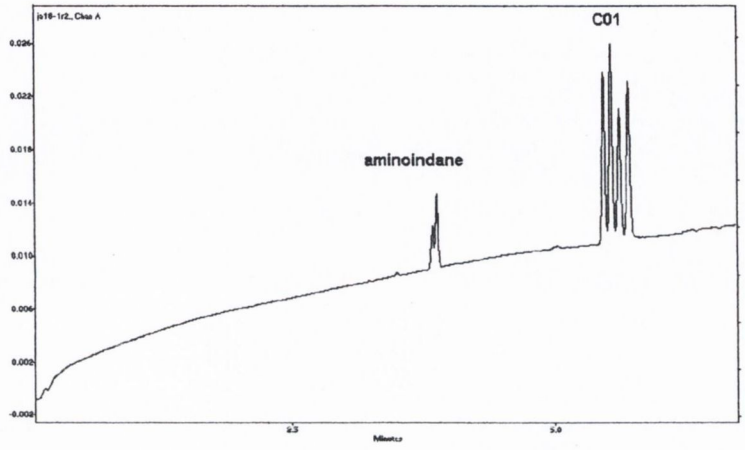


Figure 2.8: Electrophoregram of a solution of C01 and aminoindane

(conditions as in the text)

As with the non-chiral method, later it was found that a 100 mM phosphate buffer (pH=3) and 100 mM TBA with the same combination of CDs, provides a more robust method.

The same method separates the optical isomers of **C04** ($Rs_{1,2}$=1.0, $Rs_{2,3}$=2.1, $Rs_{3,4}$=2.6) (Figure 2.9), **C05** (Rs=0.97), and **C02** (Rs=0.80). For **C03,** a mixture of 10 mM of β-CD, 60 mM of HP-β-CD and 40 mM of DM-β-CD was necessary in order to achieve separation of the two enantiomers (Rs=1.02).

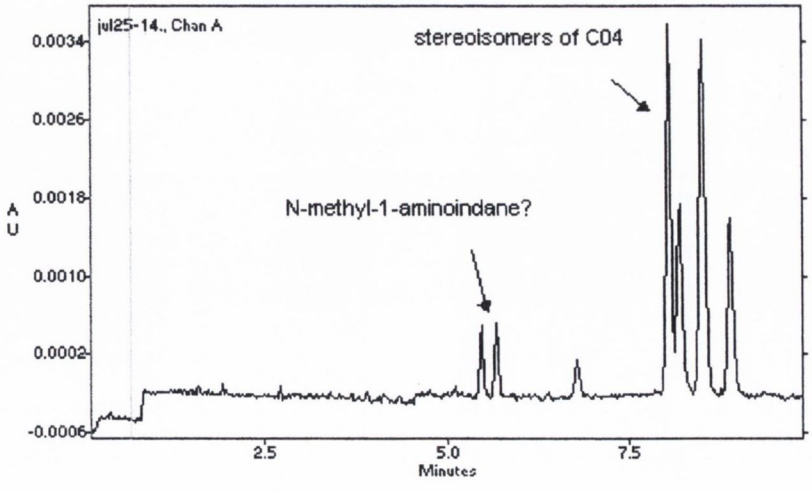


Figure 2.9: Separation of the stereoisomers of compound C04

Compound **C10** should be constituted by 8 isomers. A running buffer containing 10mM of β-CD, 40 mM of DM-β-CD and 50 mM of HP-β-CD afforded the best resolution with separation of seven peaks (Figure 2.10). It was impossible to resolve the eight isomers.

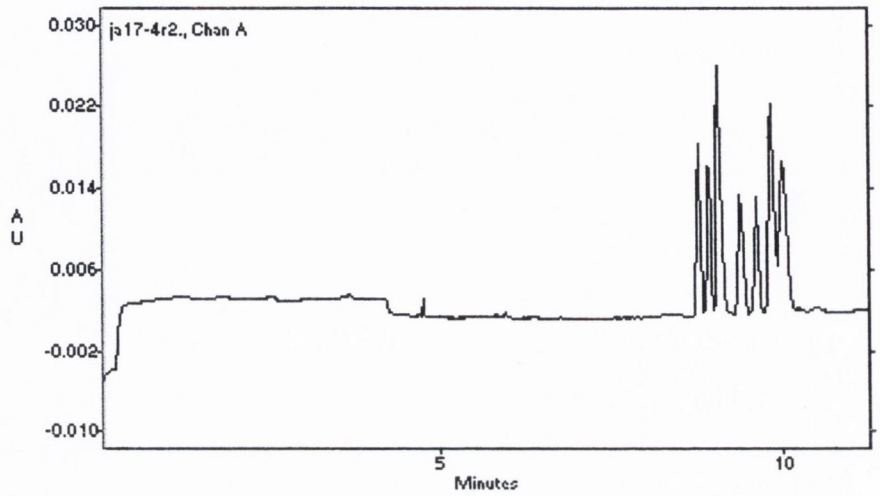


Figure 2.10: Separation of the stereo isomers of C10 by CE

(for conditions see text)

Amides **C08**, **C07** and **C09** cannot be analysed by CE using the low pH buffers containing TBA because they are neutral species and cannot move towards the cathode in the presence of an EOF suppresser. However, migration might be possible in the absence of TBA, as some EOF usually exists even at low pHs. Nevertheless, separation of these compounds from each other can only be achieved if a charge can be imparted to them by the use of a surfactant, for example. Moreover, to attain stereoresolution, a chiral selector has to be used. For this reason, several basic running buffers were tested in the presence of SDS and neutral CDs.

SDS can only be used at pH>5 with direct polarity, because at lower pHs the mobility of the micelles is higher than the EOF. A 10 mM borax buffer containing 100 mM of SDS used at constant current of 175 μA was used with a 20 cm capillary. The three amides had migration times of approximately seven minutes. Addition of methanol (5% v/v) reduces the migration times to about four minutes. The addition of DM-β-CD and/or HP-β-CD did not promote the resolution of the stereo isomers of any compound.

Replacement of the borax buffer by a phosphate buffer pH=7 improved sensitivity. Amongst several systems tested, adjoining peaks were produced for **C07** only with a 50 mM phosphate buffer containing 50 mM SDS and 50 mM TM-β-CD. Adjoining peaks were also obtained from **C02**, **C01** and **C10** when TM-β-CD was replaced by DM-β-CD.

Chiral charged surfactants like sodium taurodeoxylcholate (STDC, Figure 2.11[161]) have been used to impart mobility and stereoselectivity simultaneously. STDC can be used at a wide range of pHs as it is ionised even at low pHs.

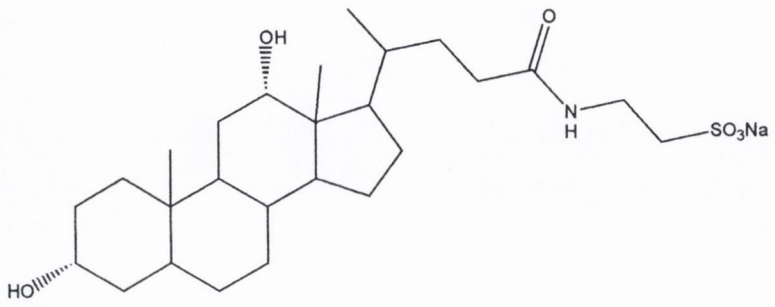


Figure 2.11: Structure of sodium taurodeoxycholate

STDC was tested at different concentrations, pHs and combinations with cyclodextrins. The amides **C07**, **C09** and **C08** were tested as well as compounds **C01** and **C05** as representatives of the amines.

A 10 mM borax buffer containing 45 mM of STDC (at 30 kV) separates two peaks of **C01** (Rs=1.5). By addition of 20 mM of DM-β-CD it was also possible to separate the two enantiomers of **C05** (Rs=0.86). Compounds **C07**, **C08** and **C09** had different migration times revealing interaction with the selector, but the optical isomers were not separated. The addition of HP-β-CD, DM-β-CD (15 mM), β-CD (saturation) or TM-β-CD (50 mM) also did not promote the separation of the optical isomers.

For tests at low pHs, reverse polarity was used, since the EOF might not be enough to carry the negatively charged micelles to the cathode.

In a 50 mM phosphate buffer pH=2.5, the amines demonstrated interaction with the micelles as their migration times decreased with increasing concentration of the selector (20 to 80 mM). The interaction however, must not involve the chiral centres, as the optical isomers were not separated.

Compound **C08** was not detected in 50 min. This probably means that the compound does not interact with the micelles at low pH. Amide **C09** however, interacts with the micelles at the chiral centre as the compound is partially separated into two peaks with a buffer containing 40 mM of STDC (Rs=0.7). **C07** gave puzzling results as it was not detected at high concentrations of STDC but one peak could be seen with a buffer containing 20 mM of the selector. However, a buffer containing 80 mM of STDC and 20 mM of DM-β-CD afforded partial separation of two peaks (Rs=0.9) at a longer migration time.

The presence of STDC often led to current problems and capillary blockage.

A charged CD, sulfated-β-cyclodextrin (SP-β-CD) can also provide for mobility of neutral compounds. In this case, reverse polarity has to be used as well, when the EOF is not sufficient to drag SP-β-CD to the cathode. For facility, in this case it was preferred instead to inject the samples at the detector end of the capillary, which produces the same effect. In this situation, the effective length of the capillary is only 12 cm.

If the test compounds interact with the selector, they can be transported towards the anode. An increase in the selector concentration should account for a decrease in the migration times as the adduct compound-SP-β-CD will be negatively charged and consequently will move faster than the free compound.

Concentrations of 0.5-5% of SP-β-CD in 50 mM phosphate buffer (pH=2.5) separated the four enantiomers of **C01** and the two 1-aminoindane enantiomers (Figure 2.12, 25kV; 12 cm effective lenght).

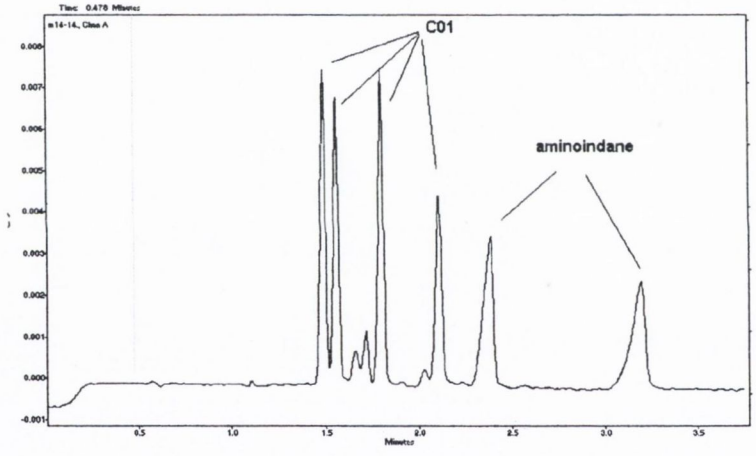


Figure 2.12: Chiral separation of C01 and 1-aminoindane with SP-β-CD (5%)

It was in fact observed that an increase in the concentration of SP-β-CD produces a decrease in the migration times when working at constant voltage. However, at constant current, the reverse is observed. This may be due to an effect on the ionic strength of the running buffer that reduces the voltage necessary to achieve the same current and consequently, the EOF. With a reduced EOF, SP-β-CD and its adducts cannot travel faster to the anode. The same effect had also been noted with different concentrations of the phosphate buffer.

The two enantiomers of **C02** (Rs=14) and **C03** (Rs=1) were also separated by SP-β-CD. However, when compared with the systems that involve the use of neutral CDs, this system has lower sensitivity and is not as robust to changes in the composition of the sample matrix in terms of pH and/or ionic strength.

Compounds **C08** and **C11** were not resolved by any of the tested systems. There were no conclusive results from compound **C06**. This was due to the fact that the compound degrades very quickly in solution and that was not known at this stage of the work.

Table 2.1 provides information on the conditions that afford better resolution (even if only partial) of the test compounds with different selectors.

Table 2.1: Detection and resolution of the different compounds in the presence of different chiral selectors (see text for labels).

	buffer	pH	capillary	Volt/curr	selector	modifiers	Polarity
1-am	Phos. (100 mM)	3	20 cm	150 μA	15 mM HP-β-CD, 15 mM DM-β-CD	100 mM TBA	direct
	Phos. (50 mM)	2.5	12 cm	50 μA	SP-β-CD 5%		reverse
C01	Phos. (100 mM)	3	20 cm	150 μA	15 mM HP-β-CD, 15 mM DM-β-CD	100 mM TBA	direct
	Borax (10 mM)	9.5	50 cm	30 KV	45 mM STDC		direct
	Phos. (50 mM)	2.5	12 cm	50 μA	SP-β-CD 5%		reverse
C02	Phos. (100 mM)	3	20 cm	150 μA	15 mM HP-β-CD, 15 mM DM-β-CD	100 mM TBA	direct
	Phos. (50 mM)	2.5	12 cm	50 μA	SP-β-CD 5%		reverse
C03	Phos. (50 mM)	2.5	20 cm	150 μA	10mM β-CD 60 mM HP-β-CD, 40 mM DM-β-CD	150 mM TBA	direct
	Phos. (50 mM)	2.5	12 cm	50 μA	SP-β-CD 5%		reverse
C04	Phos. (100 mM)	3	20 cm	150 μA	15 mM HP-β-CD, 15 mM DM-β-CD	100 mM TBA	direct
C05	Phos. (100 mM)	3	20 cm	150 μA	15 mM HP-β-CD, 15 mM DM-β-CD	100 mM TBA	direct
	Borax (10 mM)	9.5	50 cm	20 KV	20 mM DM-β-CD, 45 mM STDC		direct
C07	Phos. (50 mM)	2.5	50 cm	20 KV	20 mM DM-β-CD, 80 mM STDC		reverse
	Phos. (50 mM)	7	50 cm	20 KV	50 mM TM-β-CD	50mM SDS	direct
C09	Phos. (50 mM)	2.5	50 cm	20 KV	40 mM STDC		reverse
C10	Phos. (50 mM)	2.5	20 cm	150 μA	10mM β-CD 50 mM HP-β-CD, 40 mM DM-β-CD	150mM TBA	direct

In general, TBA was necessary to achieve better peak shapes and resolution when CDs were used. Mixtures of HP-β-CD and DP-β-CD afforded resolution of most compounds. Typically, with 20 cm or shorter capillaries, run times of less that 10 minutes afforded separation of the stereoisomers of the test compounds.

2.3.2. HPLC

2.3.2.1. Reverse phase (C18)

A C18 column was used to attempt the separation of the two diastereomers of **C01**.

A mobile phase containing 37.5 % v/v methanol (MeOH), 0.3% v/v of phosphoric acid and 0.1% (v/v) of triethylamine in water (1.5 ml/min) was able to separate the two compounds. The retention times were 7.2 and 8.6 min. This mobile phase also allows the separation of 1-aminoindane (3.3 min), 3-hydroxyindanone (4.8 min) and 3-metoxyindanone (11.9 min).

In order to elute 2-inden-1-one, a gradient method had to be used, starting with the previous mentioned mobile phase for 10 min and increasing the concentration of methanol to 90% in 20 min.

2.3.2.2. Chiral HPLC

As in CE, chiral selectors like cyclodextrins (amongst others) can be added to mobile phases to achieve stereo resolution by HPLC with non-chiral columns. However, due to the large amounts of solvents used, this method is not as economically attractive in the case of HPLC as it is in CE. For this reason, the use of a chiral column may be a better option despite the necessary initial investment.

These two cases fall in the direct chiral methods category, which involves the production of diastereomeric adducts with the analytes, on the mobile phase or on the stationary phase, respectively.

Another possibility (usually referred as the indirect method) consists in the pre-column derivatisation of enantiomers to produce diastereomers that can be easily separated with non-chiral columns and mobile phases[162].

For the present work, the direct method with a Pirkle type stationary phase (SP) was chosen due to the versatility of these SPs in being able to resolve enantiomers of a wide range of compounds[163].

Pirkle SPs contain strands of chiral selectors connected to silica. The strands possess π-donor or π-acceptor aromatic fragments as well as a hydrogen bonding agent and a dipole stacking inducing structure. These SPs are adequate to resolve analytes that contain a hydrogen bond acceptor and a conjugated π-system[163].

In these "brush-type" columns, one enantiomer is supposed to intercalate a portion of its structure between adjacent selector strands to a greater extent than the antipode does[164].

Different π-donor and π-acceptor SPs were initially prepared but the most versatile column of this type, Whelk-O 1 (see Figure 2.13), contains both structures in the same strand and for this reason is able to resolve a variety of analytes containing either electron-rich or electron-deficient aromatic systems.

The "three-point-interaction model" applies to this SP. This means that three interactions are necessary, between the SP and the analyte and one of these interactions has to be different for each isomer. This different interaction affords chiral discrimination whereas the other two contribute for retention[165].

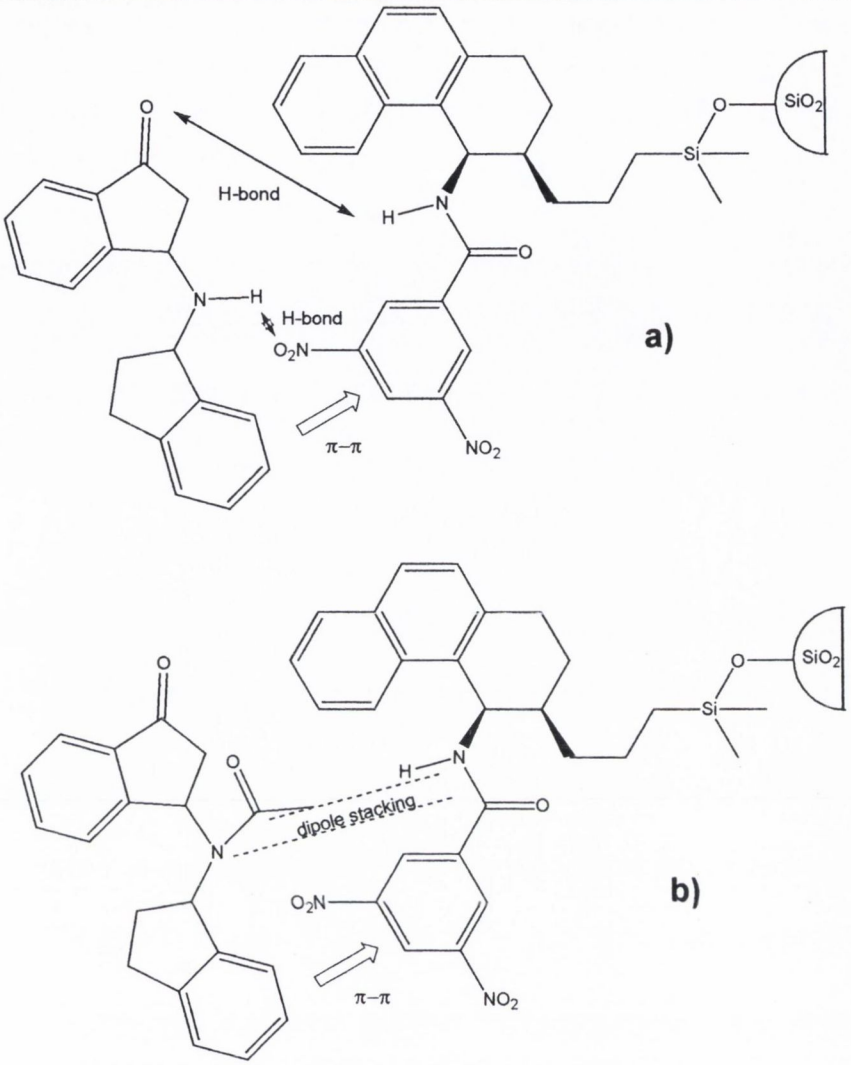


Figure 2.13: Possible chiral recognition of the test compounds C01 and C07 with (*R*,*R*) Whelk-O 1, by hydrogen bond (a) and dipole stacking (b) mechanisms

Figure 2.13 illustrates two possible mechanisms of recognition of the test compounds. An amine group next to the chiral centre may account for stereo recognition by hydrogen bonding, while amides may induce dipole stacking with the amide portion of the chiral selector. In both cases, π-π interaction is possible with the π-acceptor group of the SP.

Traditionally, basic amino compounds were not well resolved by this column owing to the very strong retention of these compounds, however this problem can be overcome by the introduction of small amounts of a polar modifier like triethylamine[163].

This column allows the use of both normal and reverse phase eluents. The two systems were attempted for the separation of the test compounds.

a) Normal phase

In the normal mode phase and with mobile phases consisting of 2-3% (v/v) of isopropanol (IPA), 0.2-0.3% (v/v) of acetic acid and 0-0.5% (v/v) of triethylamine (TEA) in hexane, large peaks were obtained, for the β-aminoketones and their shapes appeared to be slightly improved by increasing the concentration of TEA. However this system had also problems of high pressure in the column and afforded peaks with large tails.

Mobile phases containing up to 30% (v/v) of IPA in hexane and further modified with up to 5% (v/v) of acetonitrile (ACN) did not promote better separations for the amines but partially resolved the isomers of compounds **C07**, **C08** and **C09** (Figure 2.14, : 10% (v/v) IPA, 1.8% (v/v) ACN in hexane; 1 ml/min, 240 nm).

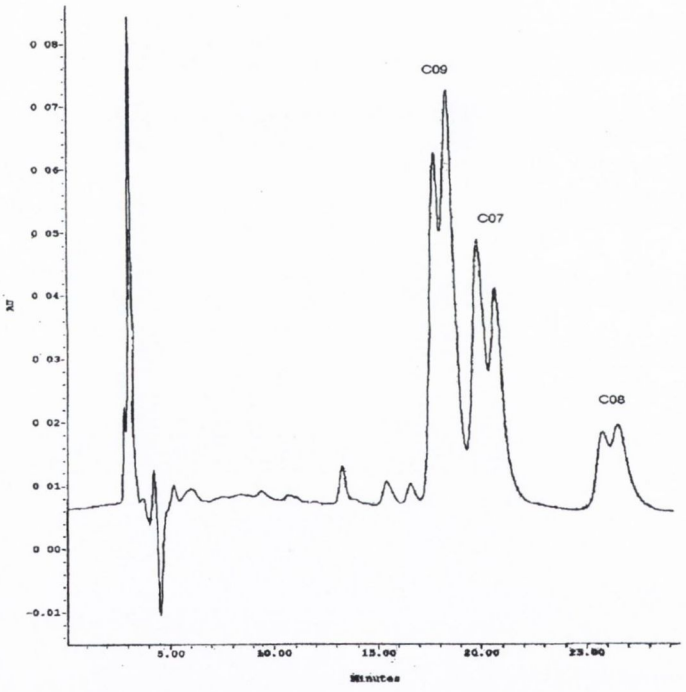


Figure 2.14 Partial resolution of isomers of compounds C09, C07 and C08 in a Whelk-O 1 column with a normal phase eluent

b) Reverse phase

With a mobile phase containing 25% (v/v) of MeOH and 0.05% (v/v) of acetic acid in water two peaks were obtained from a sample of **C01** at 18 and 20 min (0.8 ml/min). Increasing the percentage of MeOH to 35% (v/v) and the flow to 1 ml/min, the retention times of the two peaks were reduced to 8.2 and 9 min. When triethylamine was added to the mobile phase (0.1% v/v), another two large and asymmetrical peaks appeared between 19 and 23

min. The first two peaks were unaffected and were later identified as the stereoisomers of 1-aminoindane. The second pair of peaks was due to the test compound.

Modification of the mobile phase by manipulation of the relative concentrations of its components and by adding acetonitrile and tetrahydrofuran (THF), provided slightly better shaped peaks. However, not more than two peaks were separated from **C01** with these methanol based mobile phases.

The replacement of methanol, by smaller quantities of IPA and ACN, partially resolved the enantiomers of **C01**. The best mobile phase containing these components consisted of 0.95% (v/v) TEA, 0.95% (v/v) phosphoric acid, 8% (v/v) IPA and 3% (v/v) ACN in water and afforded the chromatogram depicted in Figure 2.15. Replacement of phosphoric acid by acetic acid or IPA by THF or n-propanol did not provide better separations.

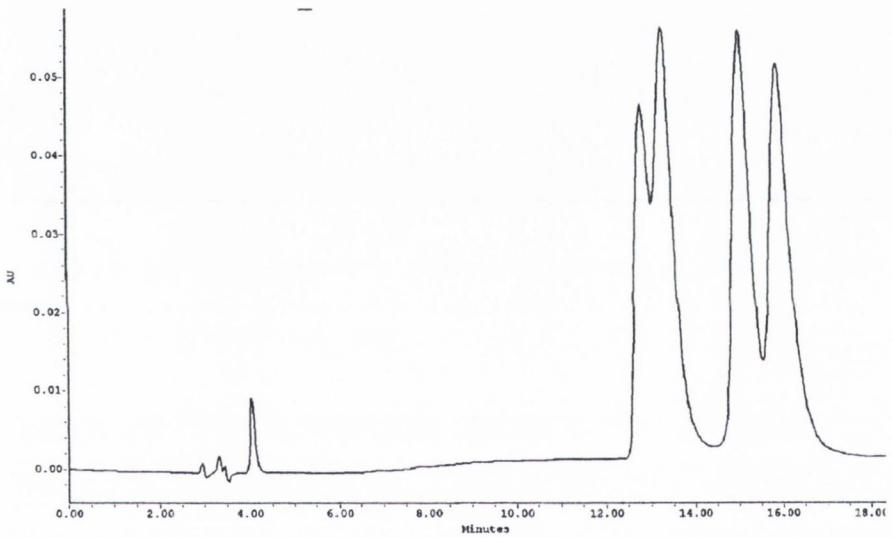


Figure 2.15: Improvement in the separation of the enantiomers of C01 with a mobile phase containing IPA (for conditions see text)

IPA was then replaced by 2-butanol which also afforded partial separation of the four enantiomers of **C01**. The relative concentrations of the components of the mobile phase were varied until the best possible resolution was obtained. It was found that TEA was necessary to maintain the shape of the peaks and a good resolution but the pH has to be kept low in order to attain feasible retention times. For that reason, the percentage of these two components has to be fairly similar. The best composition found consisted of 3% (v/v) ACN, 2% (v/v) 2-butanol, 2% (v/v) phosphoric acid, 3% (v/v) TEA in water.

This mobile phase also allowed the separation of the enantiomers of 3-methoxyindanone, and compound **C11** (Figure 2.16). However, a stronger acidic buffer, capable of

maintaining the pH≈3 in the presence of TEA could probably afford a better resolution and a more robust method.

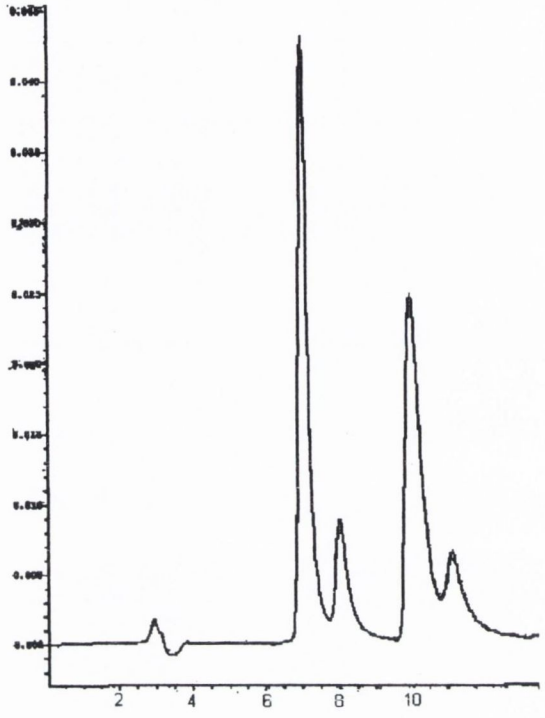


Figure 2.16: Separation of the isomers of compound C11 by HPLC

2.4. Identification of isomers of C01 by X-ray crystallography and molecular modelling

Identification of stereoisomers is necessary for the study of individual activities, toxicities and metabolism. X-ray crystallography was used at the ITQB, Oeiras, Portugal, for the establishment of the configuration of one of the enantiomers of **C01**. Configurations of the other isomers were derived from it.

2.4.1. Assignment of each isomer of C01 to its order of elution from CE and HPLC

Individual isomers of **C01** were prepared as described in 2.2. The identification of the last eluting isomer (flash chromatography) prepared with *S*-1-aminoindane was established by X-ray crystallography as the isomer 3-(*S*-indan-1-ylamino)-*R*-indan-1-one (*R,S*-**C01**). Its spatial arrangement structure is depicted in Figure 2.17. This compound corresponded to the peak with the highest migration time detected by chiral CE (15 mM HP-β-CD, 15 mM DM-β-CD).

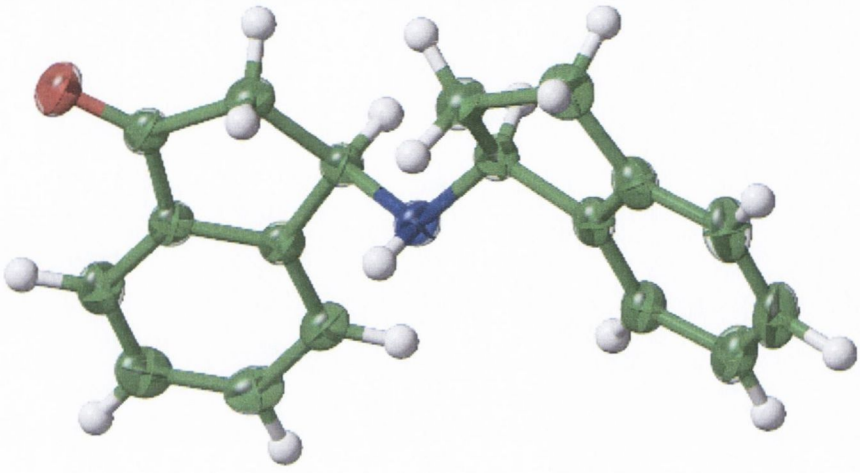


Figure 2.17: Crystallographic structure of 3-(*S*-indan-1-ylamino)-*R*-indan-1-one

Based on this, the first eluting isomer (flash chromatography) obtained in the same reaction had to be 3-(*S*-indan-1-ylamino)-*S*-indan-1-one. This was the compound corresponding to the second eluting peak observed by CE.

The two diastereomers obtained in a batch of **C01** prepared from *R*-1-aminoindane were separated by flash chromatography. The first fraction contained the diastereomer *R,R* (which is the enantiomer of *S,S*) and the second fraction contained diastereomer *S,R* (the enantiomer of *R,S*). Based on this and on the already known identification of the peaks of isomers *R,S* and *S,S*, the order of migration of isomers *S,R* and *R,R* could be assigned (Figure 2.18).

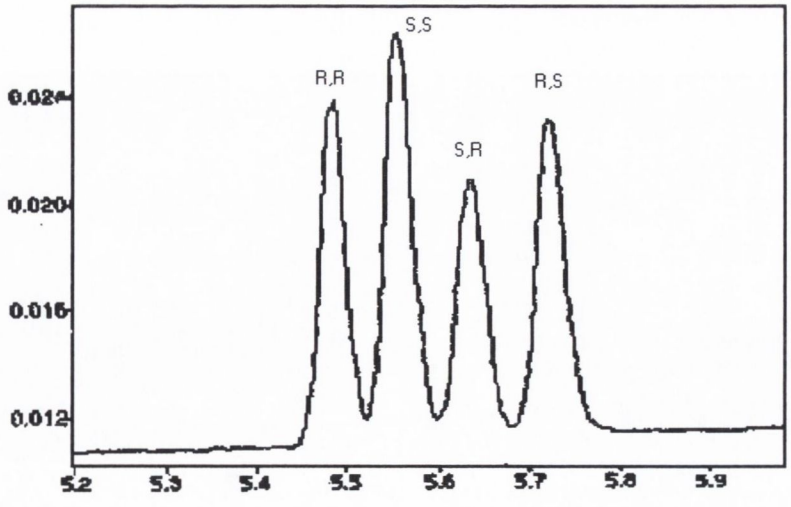


Figure 2.18: Order of migration of the isomers of C01 in CE

The order of migration was found to be the same in chiral HPLC (Figure 2.19).

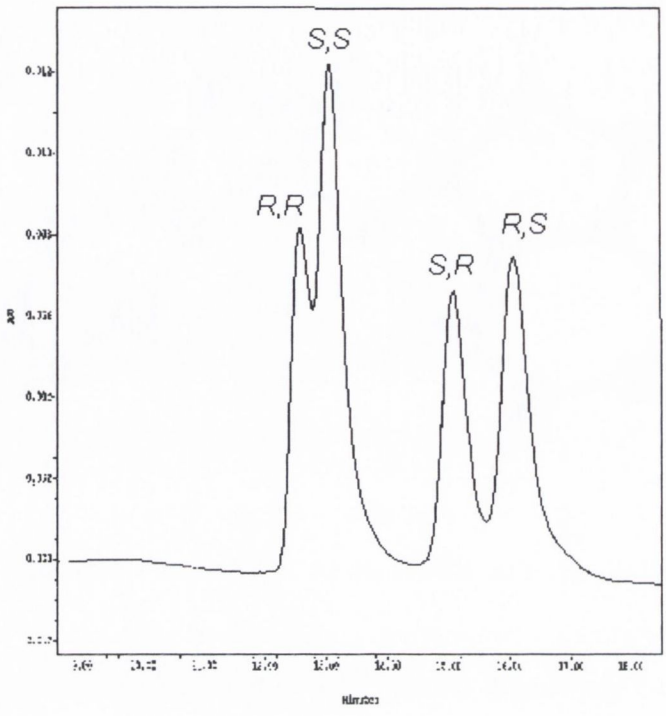


Figure 2.19: Order of migration of the isomers of C01 in HPLC

2.4.2. Conformation of each isomer of C01 as determined by molecular modelling

Based on the structure of the isomer analysed by X-ray diffraction (3-(*S*-indan-1-ylamino)-*R*-indan-1-one, Figure 2.17), the spatial arrangements of the others were inferred by molecular modelling (Figure 2.20).

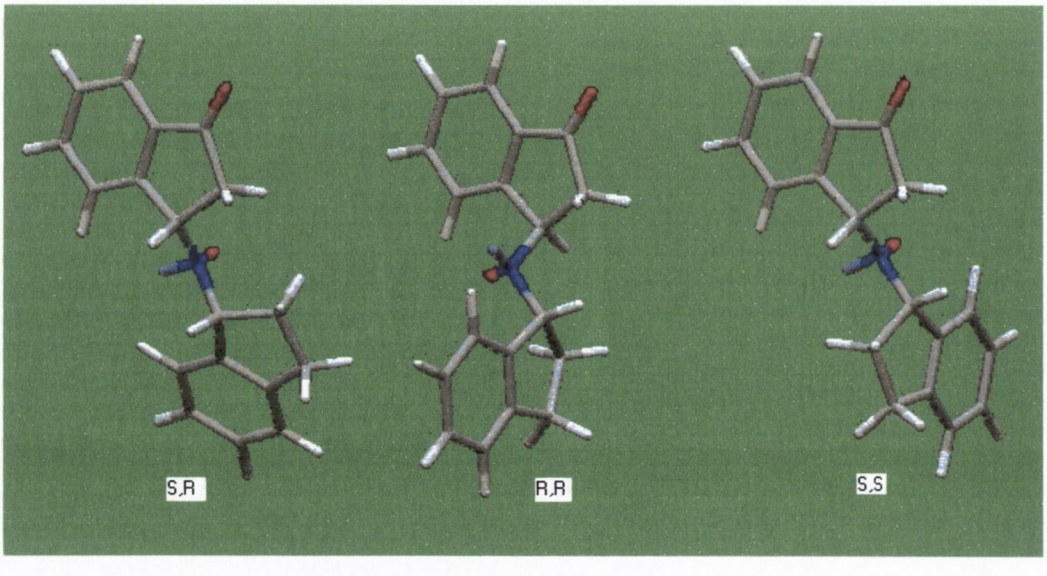


Figure 2.20: Molecular modelling of other structures of C01

2.5. P450 metabolism

When a xenobiotic enters the body, a series of non-specific enzymes transforms it in such a way as to make it more polar and consequently more easily eliminated through the normal bodily processes. While this is a highly desirable defence mechanism against the toxic materials that we are exposed to every day, it is not as welcome when the foreign material is a drug that has to be retained in the body for a certain time and reach a certain organ in order to be effective. Also, as a result of metabolism, other toxic compounds may be produced and this has to be investigated during the course of drug development.

Although every tissue has some ability to metabolise drugs, the liver is the principal organ for drug metabolism. However, following oral administration, some drugs are more extensively metabolised in the gastrointestinal tract[166].

This overall process that occurs before the drugs reach the systemic circulation is called first pass metabolism and it may so greatly limit the bioavailability of orally administered drugs, that other routes of administration may have to be used.

The cytochrome P450 system is responsible for most first-pass metabolism[167]. It consists in a group of more that 25 related enzymes, which work mostly in the liver but there are also some in the small intestine. For this reason, test of metabolism by this system is an important step in drug development.

In general, cytochrome P450 catalyses either hydroxylation or epoxidation of various substrates. Amines can be substrates for cytochrome P450 as depicted in Figure 2.21[166].

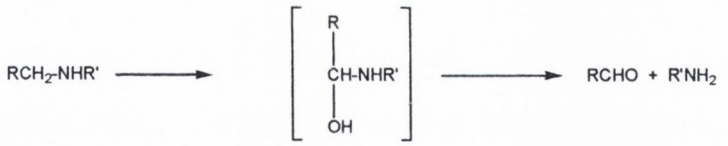


Figure 2.21: Amines as substrates for cytochrome P450

Metabolism of some of the test compounds due to cytochrome P450 was tested with Supermix™.

Supermix™ is a mixture of the six principal cytochrome P450 enzymes in which the activity of each individual enzyme is proportional to the their activity in human liver microsomes.

Metabolism of **C01**, **C03**, **C06** and **C09** was tested for 1 and 2 hours. Control samples (in the absence of Supermix™ or in the absence of substract) were analysed simultaneously. Section 7.3 describes the general procedures used.

Initially, **C01** was tested at concentrations of 100 and 200 mM for 1 or 2 hours. 25 or 50 μl of each sample stock solution in methanol were diluted in 400 μl of sodium chloride (250 mM) and injected by pressure for 20 seconds in the capillary electrophoresis. A chiral phosphate (50 mM) running buffer pH=2.5, containing 50 mM of TBA, 15 mM of HP-β-CD and 15 mM of DM-β-CD was used.

In the presence of Supermix™ most samples had peak areas smaller than control samples, generally between 2 and 21%. However, in some cases, an increase was noted (cf. Table 2.2). The samples were also analysed by chiral HPLC. No different peaks were detected in the Supermix™ samples when compared with the control samples, in CE or HPLC.

Table 2.2: Change in the areas of the peaks of the isomers of C01 in the presence of Supermix™ when compared with control samples

Conc. (mM)	Time (h)	RR	SS	SR	RS	RR+SS	SR+RS	Total
100	1	+9%	+6%	-12%	+5%	+7%	-3%	+2%
200	1	-11%	-5%	-9%	-7%	-9%	-8%	-8%
100	2	-15%	-8%	-21%	-2%	-7%	-13%	-12%
200	2	-5%	-1%	-10%	-3%	-3%	-6%	-5%

Althoug some metabolism may have happen, the differences observed in the samples incubated for 1h in comparison with the ones incubated for 2 hours are not conclusive particularly due to the results relative to 200 mM concentration. This led us to question if the assay was actually functioning properly.

A test was made with a sample of testosterone in the presence of P450, as a positive control, and compared with an assay in the presence of CYP3A4, which is the enzyme, present in Supermix™, that metabolises testosterone to 6-β-hydroxy testosterone. The expected response in Supermix™ should be proportional to the percentage of CYP3A4 that it contains. The results of this test, in terms of the amount of 6-β-hydroxy testosterone produced with both assays, are presented in Table 2.3.

Table 2.3: Positive test of the assay with Supermix

	Expected (pmol/mg/min)	Produced (pmol/mg/min)	difference
control	0	0	
P450	6620	5700	-14%
CYP3A4	22000	22500	+2.3%

Despite the difference of 14% between the expected and the obtained amount of 6-HT from Supermix™, the positive test was still considered successful, as, if P450 is able to metabolise **C01**, it should still be noticed.

Further tests were done for **C01** (200 and 400 mM; 1 and 2 h), **C03**, **C06** and **C09** (200 mM, 1 and 2 h). Samples were analysed by non chiral HPLC.

The tests with **C01** confirmed the previous ones. The presence of 1-aminoindane and another peak that was later identified as 2-inden-1-one, was noted in both controls and samples in the presence of Supermix™. Differences between control samples after 1 and 2 hours suggested some chemical degradation was occurring and this was confirmed later.

Compound **C06** was completely degraded in both control and enzyme samples after 1 hour and for this reason no conclusions could be taken. No metabolism was noted in compounds **C03** and **C09**.

2.6. Degradation

During method development, it was observed that in samples of β-aminoketones, the corresponding amines were also usually present. Moreover, the analysis of the tertiary β-aminoketones was difficult with unreproducible results and ghost peaks often appearing. It was also noticed that the same sample solution of **C01** in phosphate buffer (pH=7.4)/ACN, analysed in two consecutive days, in the same conditions, originated peaks with smaller areas on the second day (Figure 2.22).

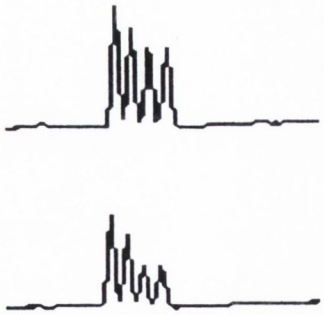


Figure 2.22: Degradation of a solution of C01 in a 24h period

The effect was more pronounced on the R,S/S,R diastereomer with a reduction in the total area to 53% of the original against 73% in the case of the S,S/R,R diastereomer.

For these reasons it was thought that degradation could be responsible for the difficulties during analysis.

To test this hypothesis, C01 was used as a model compound to test degradation under stress conditions and storage in solution.

2.6.1. Stress tests

C01 (1mg/ml) was subjected to stress conditions in alkaline (NaOH, 0.1N), acidic (HCl, 0.1N) and oxidative (H_2O_2, 15%) solutions (30 min, reflux). Following reverse phase HPLC analyses in a C18 column it was concluded that the compound is stable in hot acidic conditions but degrades in NaOH and H_2O_2 solutions.

Figure 2.23 depicts the chromatograms of solutions of **C01** subjected to different stress conditions as well as of a solution not subjected to stress.

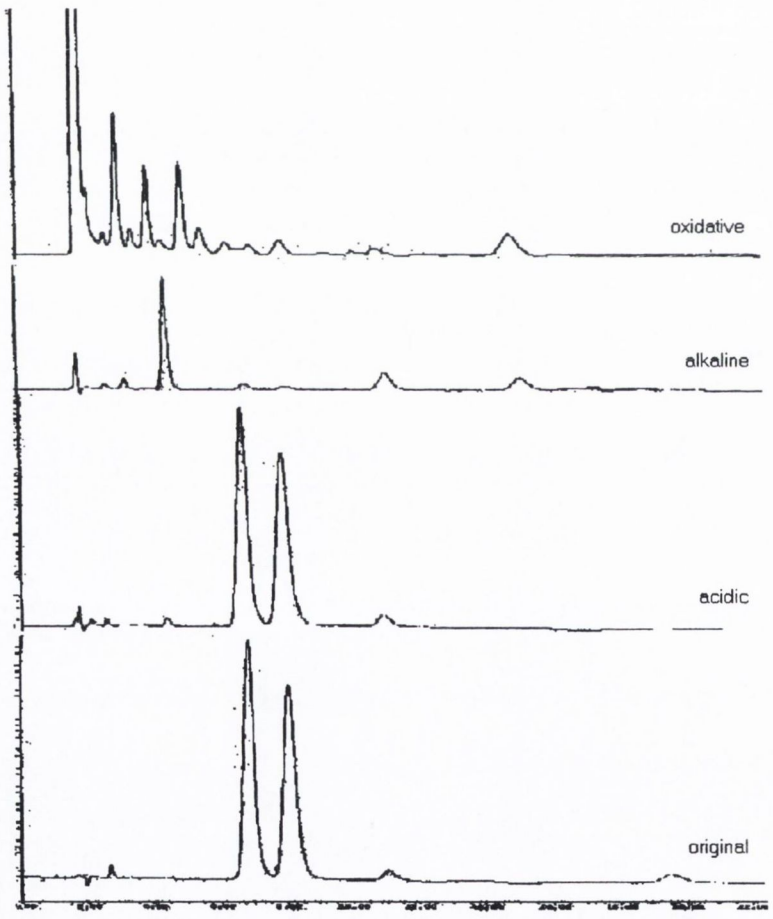


Figure 2.23: Chromatograms of a solution of C01 after being subjected to stress conditions

Chloroform extracts of each sample were analysed by GCMS but only small quantities of 1-aminoindane and 1-indanone were identified in the sample resultant from oxidative stress.

2.6.2. Long term degradation

A solution of **C01** (3.5 mg/ml) in water/methanol approx. (75:125) was kept at room temperature for one month. The solution was than extracted with chloroform and analysed by TLC, showing several spots. RP-HPLC analysis (Figure 2.24) revealed the presence of lower concentrations of the two diastereomers of the original compound (with a more significant decrease in the *S,R/R,S* diastereomer), 1-aminoindane and an unidentified peak.

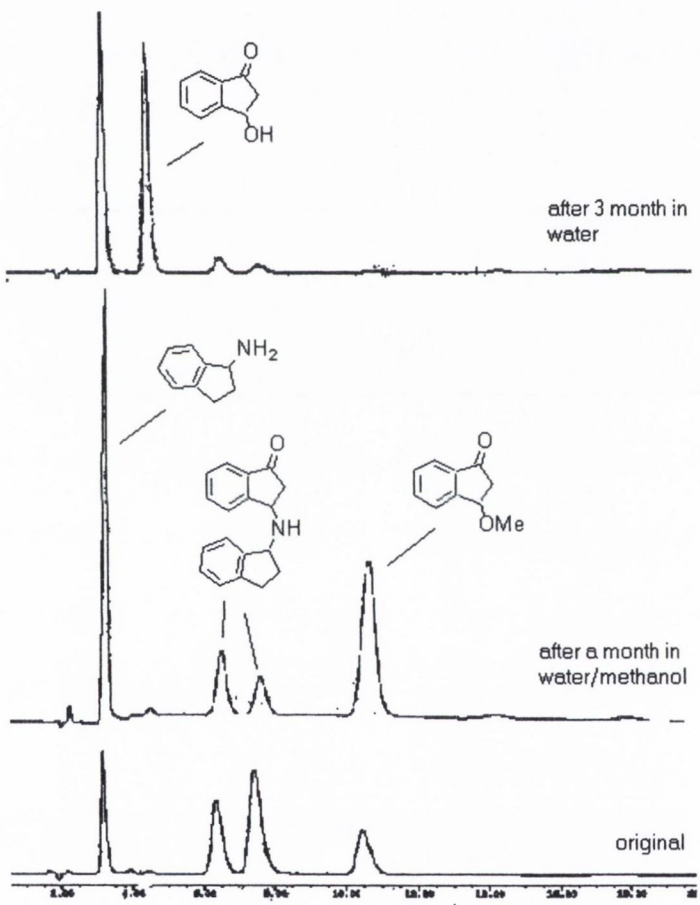


Figure 2.24: Long term degradation of C01 in water and water/methanol solutions
(214 nm, other conditions as in text)

The chloroform extract of the mixture afforded a large peak by GCMS whose mass spectrum was consistent with 3-methoxy indanone.

The extract was separated by flash chromatography using hexane/ethyl acetate (9:1) as mobile phase. The strongest spot observed by TLC proved to be 3-methoxy indanone by ^{1}H NMR (in accordance with the literature) and its retention time corresponded to the unidentified peak. The HPLC analysis using a chiral column originated two peaks, which is consistent with the presence of the two enantiomers of the compound.

Another sample of **C01** was kept in water for three months. After a few days the presence of 1-aminoindane and an unidentified peak at approximately 15 min was observed. At the end of the three-month period only 1-aminoindane and another unidentified compound, at short retention time (4.5 min) were detected by reverse phase HPLC.

2-Inden-1-one was synthesised according to the method described by Marvel and Hinman[168] using 3-bromoindanone as starting material. TEA was used instead of collidine. The retention time of 2-inden-1-one coincided with the unidentified peak at 15 min. The UV spectrums obtained by HPLC with diode array detection were also similar.

3-hydroxy indanone was synthesised by the method described by Undheim and Nielsen[169]. The retention time of 3-hydroxy indanone was 4.5 min.

It was then postulated that deamination of **C01** was occurring yielding 1-aminoindane and 2-inden-1-one (**B03**), followed by methoxylation yielding **B05** or hydroxylation yielding **B04** at the benzylic position (Figure 2.25).

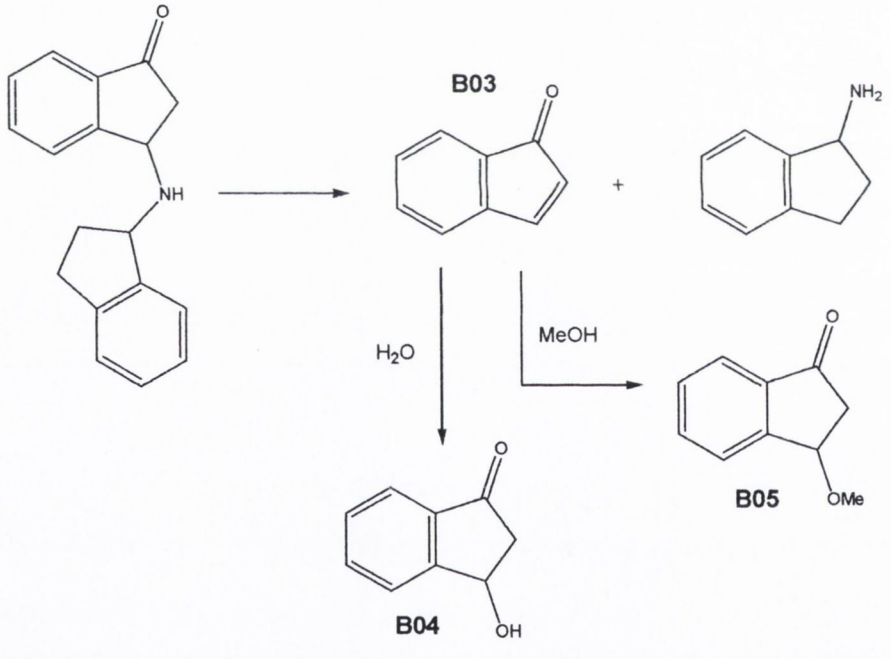


Figure 2.25: Degradation of C01 in solution

Some deamination also occurs in stock solutions in methanol (see "original", Figure 2.24, corresponding to a fresh aqueous solution that was prepared from a old stock solution in methanol. 1-aminoindane and 3-methoxy indanone were already present). Solutions in IPA and ACN are stable for longer periods.

No degradation was observed when **C10**, **C09** or **C07** were subjected to similar procedures.

2.6.3. Preliminary tests on the effect of pH on the degradation rates

Some of the compounds mentioned in section 2.1 were tested for degradation at 37°C and at different pHs. The buffers used were borate/citrate/phosphate[170] with an ionic strength of 0.05.

Initially, degradation was evaluated for **C01**, **C02**, **C03**, **C04**, **C05** and **C06** at pH 7.4 during a 4 hour period. Solutions of each compound were kept at 37°C and aliquots were assayed periodically by HPLC (non-chiral, as in section 2.3.2.1) during the 4h period. From these preliminary tests it was concluded that the tertiary amines were degrading faster than the secondary ones.

Table 2.4: Remaining percentage of each compound after 2 and 4 hours in solution at pH=7.4

Compound	% remaining after approx. 20min	% remaining after approx. 1h	% remaining after approx. 2:15 h	% remaining after approx. 4:30h
C01*		85,80	70, 57	49, 28
C02		91	75	59
C03		77	62	57
C04	47	0		
C05	25	2		
C06	11	0		

*results for each diastereomer

The non-chiral CE method mentioned in section 2.3.1.1 was then used for the evaluation of the pH/rate profiles of some of the previously mentioned compounds. The method was not individually validated for each compound, but for the surveying purpose of the analysis, this was not considered necessary at this stage of the research project. Repeatability and linearity tests were performed for some compounds and was considered adequate.

The kinetics of degradation of compounds **C05**, **C01** and **C02** were tested in citrate/phosphate/borate buffers with an ionic strength of 0.05 for pHs in the range of 2.9-11.2. The original concentrations of the compounds were 45, 55 and 62 mg/ml respectively.

During these tests it was noted that the areas of the peaks of the test compounds, when tested at low pHs, were actually increasing along time. It was thought that this was due to evaporation of the solvent in the sample vial in long period tests.

In an attempt to correct for this effect, pyridine and 2-aminobenzyl alcohol were tested as internal standards. However, pyridine was evaporating (or degrading) as well, while 2-aminobenzyl alcohol degraded at low pHs. For this reason the use of an internal standard was discarded (except for tests at different temperatures or ionic strengths at pH 7.4 where 2-aminobenzyl alcohol was stable). Correction for evaporation in pH/rate profiles was made based on the rate of increase in areas at the pHs were the compounds were stable (see Chapter 7).

The autosampler of the CE machine was connected to a water circulator and the temperature was set to achieve 37°C inside the sample vials. The buffered samples were kept in the autosampler from the first injection until the last.

The reactions were monitored for a decrease in the concentration of the original β-aminoketones. Pseudo-first-order reaction rates (K_{obs}) were calculated from the slope of linear plots of natural logarithm of remaining β-aminoketones versus time.

K_{obs} for pH 3.2 and 7.4 and respective half-lives are represented in Table 2.5. The rates of degradation are typically more than one order of magnitude larger at neutral pH than at pH=3.2.

Table 2.5: K_{obs} and half-lives at 37°C and I=0.05

Compound	K_{obs} pH=3.2 (min^{-1})	$t_{1/2}$ pH=3.2 (min)	K_{obs} pH=7.4 (min^{-1})	$t_{1/2}$ pH=7.4 (min)
C01	0.00018	3916	0.0024	285
C02	0.00007	9902	0.0014	495
C05	0.0009	770	0.0136	51

The corresponding pH/rate profiles are represented in Figure 2.26. The degradation rates of **C02** are the same order of magnitude as the degradation rates of **C01** but are in general at least ten times slower than the degradation rates of the corresponding tertiary amine **C05**.

As degradation progressed, new peaks appeared in the electrophoregrams of **C02**, **C01** and **C05.** In the case of **C02** and **C01** these peaks were identified (by migration time similarity in CE and retention time similarity in HPLC) respectively as 2- and 1-aminoindane. In the case of compound **C05**, the degradation product was thought to be N-methyl-2-aminoindane.

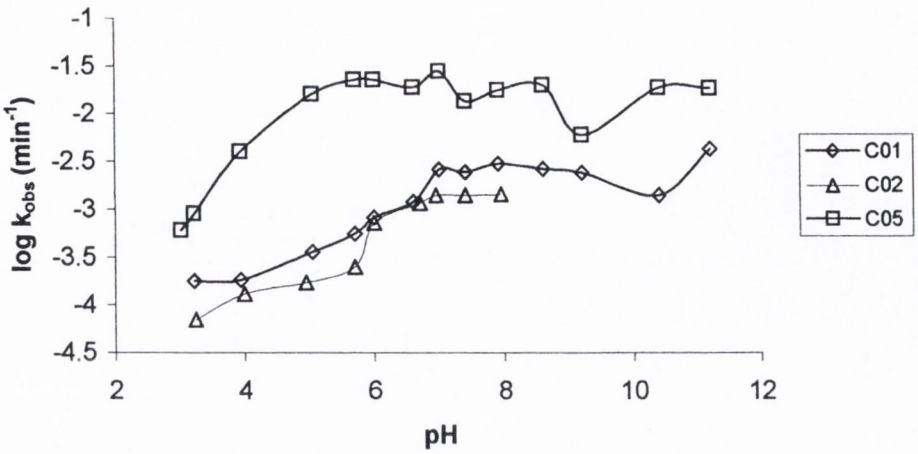


Figure 2.26: pH/rate profiles of C01, C02 and C05 at I=0.05

2.6.4. Effect of Ionic strength (I)

Degradation rates of **C01**, **C02**, **C05** were compared (pH=7.4, citrate/borate/phosphate buffers) at different ionic strengths (I=0.05, 0.1, 0.25, 0.5 and 1). Temperatures were 37°C for **C05** and **C01** and 38.5°C for **C02**. Concentrations of the compounds tested were respectively 55 mg/l for **C01**, 62 mg/l for **C02** and 70 mg/l for **C05**. 2-aminobenzyl alcohol was used as internal standard but results were not dependent of it.

Figure 2.27 shows how the logarithms of the reaction rates relate to the ionic strength for the different compounds.

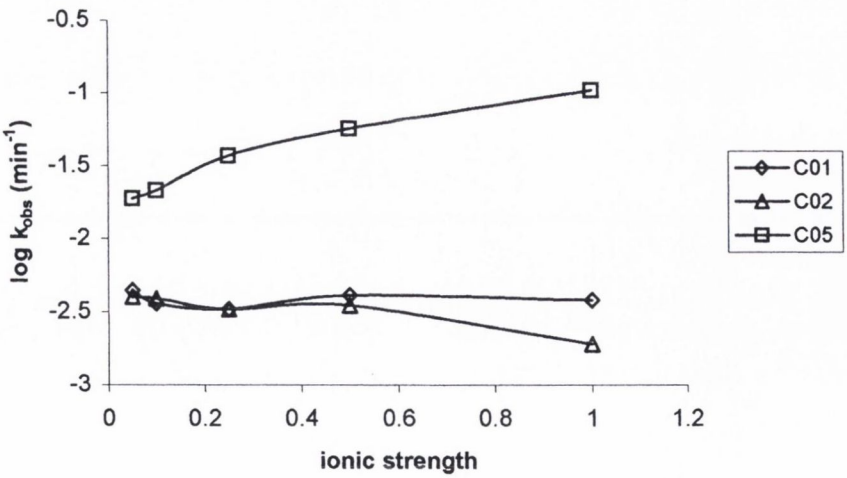


Figure 2.27: Plot of the log of the reaction rates vs. ionic strength

The rate of the reaction is higher for **C05** than for the other compounds at all ionic strengths.

For **C05** there is a direct relationship between the rate of the reaction and the ionic strength (k_{obs}=0.09x**I**+0.01, R^2=0.999). Therefore, extrapolating for I=0, the rate, in the absence of buffer, should be 0.01 min^{-1}. **C02** and **C01** appear to be less sensitive to variations in the ionic strength (coefficients of determination <0.85).

2.6.5. Effect of temperature

The effect of temperature on the reaction rate was tested for **C05** in a pH=7.4 citrate/borate/phosphate buffer with I=0.154.

According to Arrhenius, the logarithm of the rate is a linear function of the reciprocal of the temperature.

$$k_{obs} = A^{(-E_a/RT)} \qquad \textbf{(2.6)}$$

or

$$\ln k_{obs} = \ln A - E_a / RT \qquad \textbf{(2.7)}$$

A is the pre-exponential factor and represents the reaction rate at infinite temperature, *R* is the gas constant (8.3143 Joules $mole^{-1}$ K^{-1}) and E_a is the activation energy.
The degradation rate of compound **C05** was found to follow the Arrhenius law in the range of 30 to 45°C as a direct relationship (R^2=0.97) exists between the natural logarithm of k_{obs} and the reciprocal of the temperature (*A*=8.0E12 min^{-1} and E_a=85 KJ mol^{-1}, Figure 2.28).

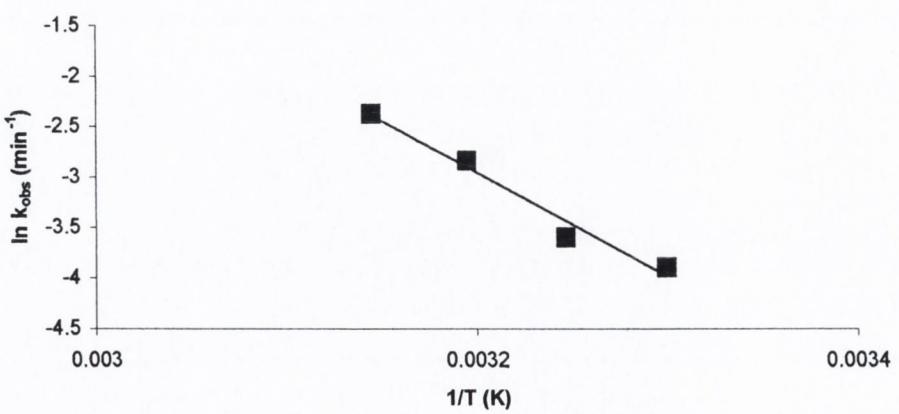


Figure 2.28: Plot of the ln of the reaction rate *vs.* the reciprocal of the temperature for C05

2.7. Conclusions

Capillary electrophoresis, using neutral CDs as chiral selectors is an adequate method for the separation of β-aminoketones derived from aminoindane or cyclopentylamine and indanone. Best overall performance was obtained with a phosphate buffer (100 mM, pH=3) containing a combination of HP-β-CD and DM-β-CD at 15 mM each. The elimination of EOF by addition of TBA (100 mM) considerably improves separation and peak symmetry. Reversal of the migration order can be achieved with other combinations of CDs.

The separation of the amides is more difficult to achieve but may be possible in some cases with the use of charged selectors.

The diversity of responses with the different systems suggests that separation with neutral CDs does not result from inclusion of the indanone part of the molecule. If it did, a general system could have been found for the separation of all compounds.

Chiral HPLC with a Pirkle I type column can also be used, the best separations being achieved in the reverse phase mode for amines and normal phase mode for amides.

Compounds **C11** and **C08** whose enantiomers were not resolved by the CE methods tested, were partially resolved by chiral HPLC.

The identification of the optical isomers of **C01** was assigned to their order of elution by flash chromatography, CE and HPLC.

Metabolism by the P450 enzymatic system does not greatly increase the rate of degradation of the β-aminoketones.

β-Aminoketones derived from 1-indanone degrade by deamination in neutral to basic aqueous solutions. Stability greatly improves in acidic conditions. The amide and alcohol derivatives are stable also in basic conditions.

A Beckman capillary electrophoresis apparatus, modified to allow the control of temperature of the sample tray, is a simple and effective system for rapid determination of the rates of degradation of these compounds.

The rates of degradation at different pHs, follow pseudo-first-order kinetics and K_{obs} generally increases with increased pH. k_{obs} may be different for different diastereomers and in some cases, is dependent on the concentration of the buffer.

Following these observations, it is possible to envisage the use of β-aminoketones as prodrug systems. The rest of the work that originated this thesis was devoted to the study of this hypothesis.

CHAPTER 3. β-AMINOINDANONES AS PRODRUGS

3.1. Introduction

During the early work described in this thesis, it was discovered that some indanone amine derivatives degrade at rates apparently suitable for application as prodrugs for amines (Figure 3.1).

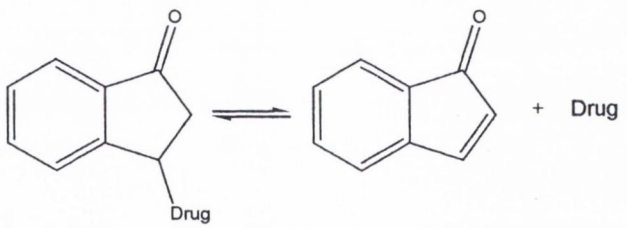


Figure 3.1: Indanone prodrug system

This chapter describes work that involved the preparation and testing of new compounds made from different model amines/drugs, in order to investigate the generality of the concept. In particular, we were interested in the relationship between decay kinetics and amine structure.

Initially, a bibliographic search was performed in order to find previous reference to the reaction involved and to ascertain that the concept had not been applied previously.

Wohl-Ziegler bromination (Figure 2.2) was then used to produce 3-bromoindanone, which was further reacted with model amines, aminoacid esters, and amine drugs to produce β-

aminoindanones (section 3.3). Model compounds were also prepared from 2-methyl,3-bromoindanone.

Critical to the success of this prodrug approach is that the amine is released at appropriate rates under physiological conditions. To investigate this, the compounds were tested for degradation over a range of pHs (section 3.4). Other conditions like temperature (37°C) and ionic strength (0.154) were set to closely resemble physiological conditions. Degradation rates of some compounds in plasma were also measured, in order to determine if there was any concomitant enzymatic metabolism. Capillary electrophoresis and/or HPLC were used to follow the decay in the concentration of the original products and the formation of the free amines.

The decay reaction was generally found to follow pseudo-first-order kinetics. However some degradation profiles could not be explained strictly by the assumption of an irreversible reaction and equilibrium had to be considered. For this reason, a small section of this chapter is devoted to a description of the theory of determination of the constant rates of reversible and irreversible reactions (section 3.4.1).

In order to investigate the reaction mechanism, the pH/degradation rate profiles were plotted and fitted by non-linear regression to an equation that considers the spontaneous degradation of the compounds in the ionised and unionised form (section 3.7).

Capillary electrophoresis was selected as the best method for the determination of the pK_as of these unstable compounds. This determination was important to further confirm the adequacy of the spontaneous degradation description, which also affords an estimation of the pK_as. The pK_as delivered by both methods were compared (section3.9).

3.2. Notes on elimination of amines from β-aminoketones, β-aminoacids and β-aminoesters

This section provides a brief review of the deamination of β-aminoketones and related compounds and previous uses of this reaction as a prodrug system.

The elimination reaction is known at least since the late XIXth century when β-alanine was already reported to degrade into acrylic acid and ammonia[171]. Around the same time, it was also observed that 1-naphthylamine was eliminated from N-1-naphthyl-aspartic acid diethyl ester in ethanol and potassium hydroxide, releasing fumaric acid[172]. Anilines were reported to be eliminated under mildly acidic conditions from β-aminoketones derived from 1,3-diphenyl propan-1-one, releasing chalcone[173]. At the turn of the century it was also noticed that the amines were easily eliminated from β-aminoketones, with formation of α,β-

unsaturated ketones, by the action of concentrated acids, acid chlorides (in benzene and potassium hydroxide), phenyl isocyanate (at 100°C) or piperidine (in ethanol)[174].

Analytical applications were later found for this reaction. The elimination of ammonia from β-aminoacids when heated with base, has been used to confirm the structure of related β-lactams[175,176].

The equilibrium between β-aminoketones, α,β-unsaturated ketones and the parent amines in alcohol solution is well known and has been extensively studied[177]. The elimination of ammonia from β-carbonyl compounds is a synthetic route to α,β-unsaturated carbonyl compounds[149]. There is however little evidence of studies on the same kind of chemistry in aqueous solution and this may be due to the relative instability of the compounds[178].

Nevertheless, the same kind of phenomenon was reported in 1956 for asparagine. By heating it at 100°C, in phosphate buffer at pH 6.7 (Figure 3.2), two degradation products were formed, the first being aspartic acid. The second, although initially incorrectly assigned, was later found to be fumaramic acid resulting from the elimination of ammonia and formation of a double bond[179]. In asparagine the amino group is in a β position to an amide and, by its elimination, the double bond formed is conjugated with the amide and the carboxylic acid.

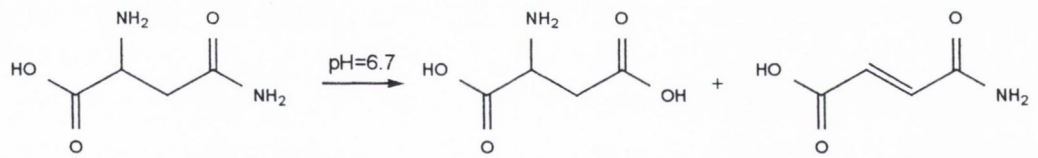


Figure 3.2: Degradation of asparagine in phosphate buffer pH=6.7

Moreover, if fumaramic acid is treated with ammonium hydroxide, asparagine is formed quantitatively while fumaric acid under the same conditions does not produce aspartic acid. Fumaric acid, in the presence of ammonium hydroxide forms the ammonium salt and the addition to the double bond is not possible, while in the case of fumaramic acid, the amide promotes the substitution of the double bond[179].

Although there are few references describing this type of base catalysed elimination in acids, this type of cleavage can be enzymatically produced in aspartic acid by 3-methylaspartase. The mechanism involves the initial removal of an α-proton before the N-C bond cleavage[180,181].

β-Aminoketones belong to a class of compounds generally known as Mannich bases which consist of an amine connected to a substrate by a CH_2 group (cf. section 4.1). In particular, β-aminoketones are C-Mannich bases. For their synthesis, a methylketone is used instead

of an amide and a C-C bond is formed, instead of the N-C bond formed in the N-Mannich reaction described previously in the context of prodrugs for amines.

Elimination from Mannich bases is well documented. Elimination may occur on either the substrate and/or the amine side, termed deaminomethylation (**a**) or deamination (**b**) respectively (Figure 3.3). Usually one process prevails over the other, depending on the compound[182].

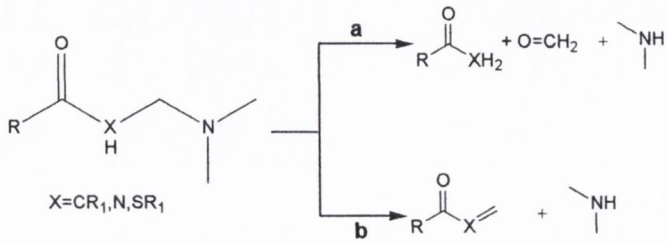


Figure 3.3: Cleavage reactions of Mannich bases

In general, C-Mannich bases, particularly the ones where the substrate is a ketone, are more stable towards deaminomethylation. Steric hindrance as well as basicity of the amine favour cleavage of Mannich bases[182].

Deaminomethylation may be considered a reverse Mannich reaction (retro-Mannich reaction) that is favoured in acidic conditions and proceeds via the imine salt with the cleavage of the C-R bond, which is the rate-determining step. It occurs predominantly when the aminomethyl group is linked to a heteroatom.

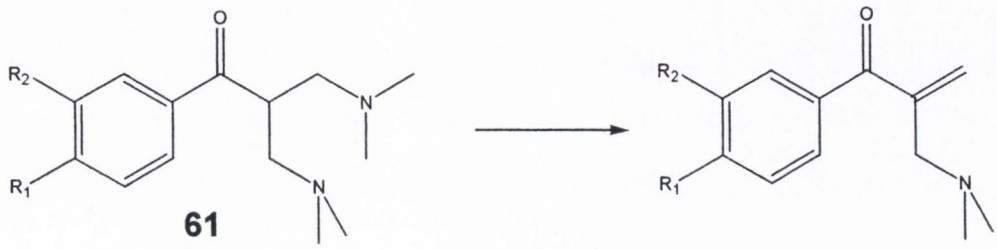
Deamination (or retro-Michael reaction) on the other hand is peculiar to C-Mannich bases. The most widely studied compounds have been the ones that afford vinyl ketones. The existence of a hydrogen atom in the α position is not essential as other groups such as a carboxylic acid or ester may also be eliminated in conjunction with the amine. [182].

The unsaturated ketone obtained by deamination can add free amine again (or other nucleophiles[183]) but not amine salts. Because of this, the reaction is said to be more complete in the presence of an equivalent of acid even though the rate of the reaction is greater in basic solutions[184].

Hetero-Mannich[182] bases, especially N-Mannich bases, and their deaminomethylation reactions have been extensively used as prodrug systems (cf. Chapter 1 and references cited therein). On the other hand, C-Mannich bases have been little investigated as prodrugs and the only examples found exploit them as prodrugs for unsaturated ketones, rather than for amines.

Some anti-microbial and cytotoxic C-Mannich bases have had their activity attributed to α,β-unsaturated ketones derived from them, which react with essential thiol groups in living organisms and inhibit enzymes from glutathione metabolism[185].

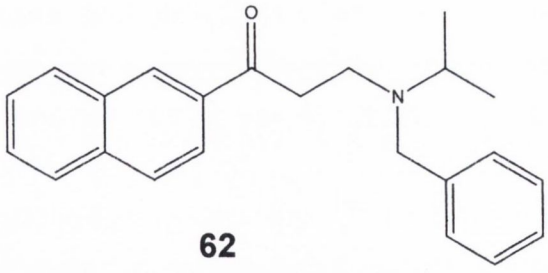
Bis-Mannich bases of acrylophenones (**61**) for example, have been shown to yield the active compound by deamination under simulated physiological conditions[186].



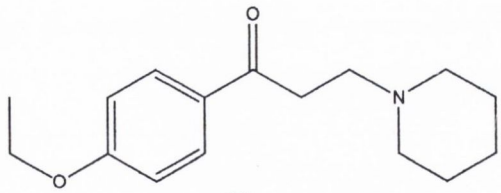
61

Figure 3.4: Prodrug system for acrylophenone by deamination of the corresponding *bis*-Mannich base

In contrast, mono amino Mannich bases derived from acetophenone are reported to be stable[187]. However, similar compounds derived from naphthyl ketones seem to undergo some elimination; compound (**62**) at pH 7.4 and 25°C, deaminates with a half-life of 36 min[188]. Falicain (**63**), which is a clinically used anaesthetic, is said to fragment via a retro-Michael reaction[189].



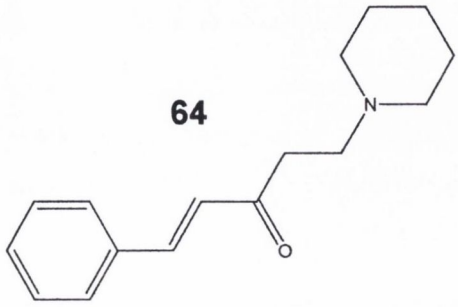
62



63

Compound (**64**) decomposes by approximately 20% when incubated at 37°C for 48h, in a mixture of 90% DMSO-d_6 and 10% deuterated phosphate buffer-saline[190].

64



In an attempt to evaluate the origin of the activity of Mannich base derivatives of ethacrynic acid, several analogues have been tested and it was proved that only the dimethylamino derivative (**65**, R_1=Et, R_2=H, X,Y=Cl) releases the compound *in vitro* (dog urine).

Substitution on the benzene ring was found to have influence, as there was no deamination if hydrogens were present instead of chlorines. In the absence of an α proton, cleavage was not possible, as expected[191].

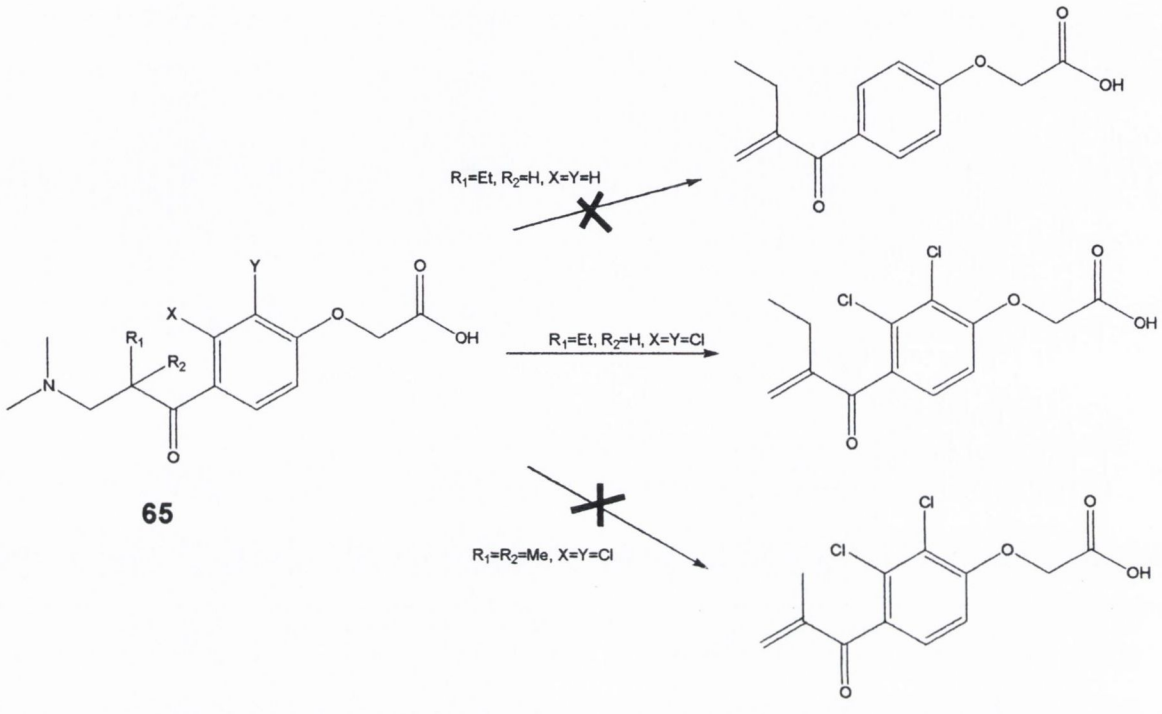


Figure 3.5: Deamination of Mannich bases of analogues of ethacrynic acid

In summary, to our knowledge, no other β-aminoketones and their deamination reactions have been described as prodrug systems. Therefore the use of derivatives of indanone as prodrugs for amines is novel and attractive based on the preliminary studies. Moreover, in comparison to N-Mannich bases, C-Mannich bases seem to have the advantage of being more stable in acidic conditions and avoiding the release of formaldehyde in physiological conditions since degradation seems to occur mostly by deamination rather than deaminomethylation.

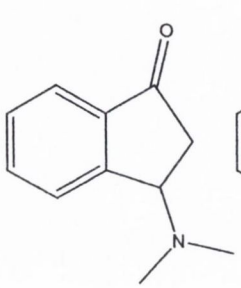
3.3. Synthesis of new test compounds

Since all target compounds were derivatives of 1-indanone and the amines were meant to be added to the benzylic position, the method of synthesis involved bromination of 1-indanone in the benzylic position (by the previously mentioned *Wohl-Ziegler* bromination, 2.2) followed by its substitution by the relevant amine.

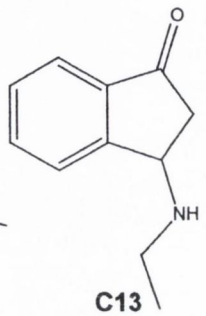
The following amines were used to obtain the corresponding β-aminoketones (the code name of each product is in brackets): dimethylamine (**C12**), ethylamine (**C13**), piperidine (**C14**) and 2-phenethylamine (**C15**) as model amines; atenolol (**C16**), tryptophan methyl

ester (**C17**), alanine methyl ester (**C18**), phenylalanine benzyl ester (**C19**), desloratadine (**C20**), and ephedrine (**C21**) as model drugs.

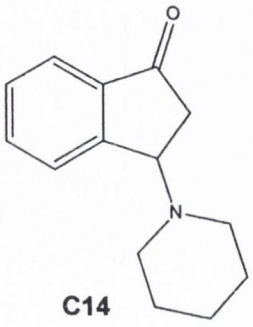
Confirmation of the identification of the final compounds was made by MS, IR and NMR (Chapter 7).



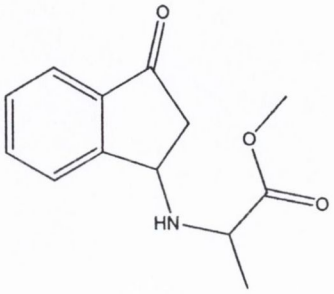
C12



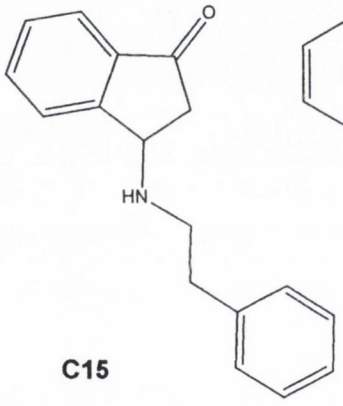
C13



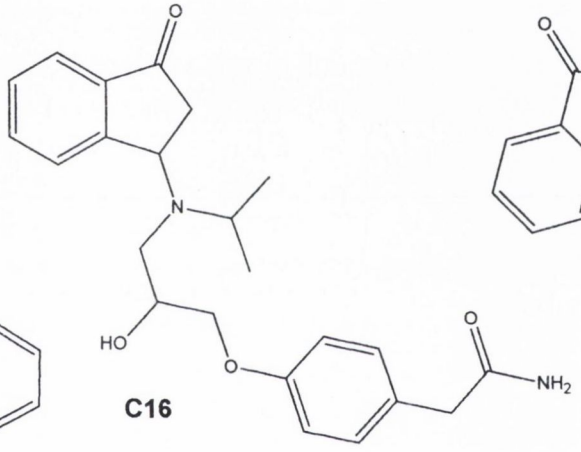
C14



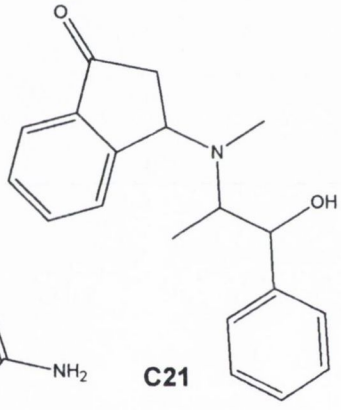
C18



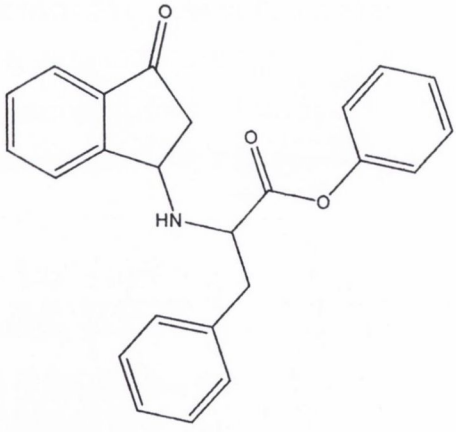
C15



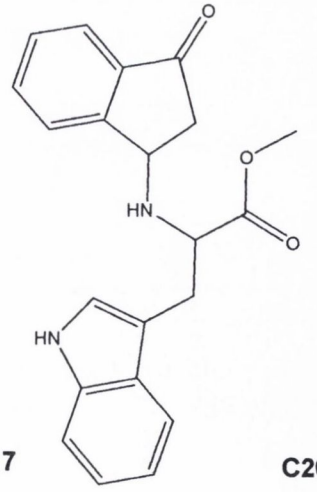
C16



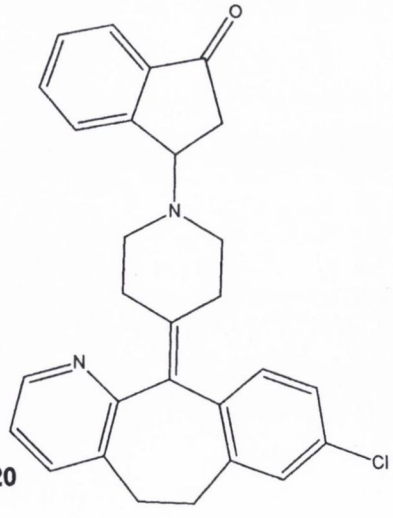
C21



C19



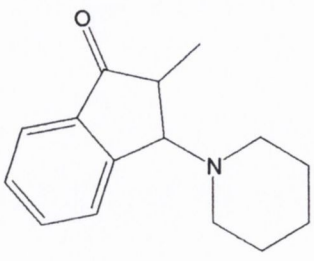
C17



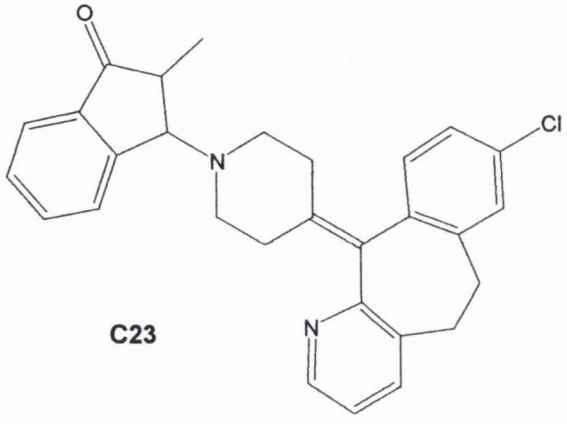
C20

Two derivatives of 2-methyl-indane-1-one were also synthesised in two steps including bromination to form 3-brome-2-methyl-indane-1-one (**B14**) followed by nucleophilic

substitution with piperidine (**C22**) and desloratadine (**C23**). This was done in order to test the effect of substitution at the ketone α-position.



C22



C23

3.4. Elimination of amines from 3-aminoindan-1-ones and 3-amino, 2-methylindan-1-ones in aqueous solution

Following the observation that ionic strength may affect the degradation rate and in order to have comparable results, it was decided to test all compounds at 37°C and consistent ionic strength of I=0.154, which simulates the physiological environment. Whenever other conditions were used, that is mentioned in the text.

Phosphate/borate/citrate buffers were used in the range of pH of 2-12[170]. The choice of buffers resulted from the possibility of preparing solutions in this range of pH, with the required ionic strength, from only two stock solutions (K_3PO_4, 0.1 M; citric/boric acid, 0.05 M/0.2 M). Moreover, buffer capacity was maximised in every case, as there was no need to add sodium chloride to set the ionic strength. For pH in the range of 0.5-2, HCl solutions were used.

Stock solutions of the test compounds of 1-10 mg/ml were prepared in acetonitrile. Aliquots were transferred to vials containing the buffers previously warmed to 37°C to produce solutions with concentrations in the range of 50 to 200 ppm. The vials were placed in the pre-warmed autosampler of the capillary electrophoresis apparatus or the HPLC system.

A non-chiral CE method was used to follow the decay in the peak areas of the original compounds when incubated at 37°C in buffered solutions, for at least two log units. For some compounds, the tests were also performed by HPLC.

3.4.1. Kinetics of elimination

In the tests on the deamination reaction of β-aminoindanones studied in the previous chapter, the elimination was found to follow pseudo-first-order kinetics. This was expected since, even if acid or basic catalysis was involved, the pH of the solution was maintained by the use of buffered solutions. Therefore this reaction can be generalised to:

$$A \xrightarrow{k_1} B + C$$

In this case A represents the β-aminoindanone, B and C represent the amine and the α,β-unsaturated ketone and k_1 is the pseudo-first-order rate coefficient.

The remaining concentration ([*A*]) of β-aminoindanone at time *t* can be described by the relation:

$$\frac{d[A]}{dt} = -k_1[A] \qquad \textbf{(3.1)}$$

Equation **3.1** can be integrated to:

$$-\ln[A] = k_1 - \ln a_0 \qquad \textbf{(3.2)}$$

where a_0 corresponds to the initial concentration of A.

This means that a plot of the natural logarithm of the remaining concentration of A agaist time, should provide a straight line. The slope of this line corresponds to the pseudo-first-order reaction rate coefficient and the concentration of A should decay to zero.

In most of the cases described in this chapter, the degradation of the compounds followed pseudo-first-order kinetics and equation **3.2** but, sometimes, a significant deviation from this type of kinetics was observed since deamination seemed to stop before all the original compound was consumed. This was attributed to the reversibility of the deamination reaction, as a reaction may not proceed to completion if the reverse reaction is possible. This means that an equilibrium is involved:

$$A \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} B + C$$

In this situation, the plot of the logarithm of the concentration of A against time does not produce a straight line, despite the fact that the hydrolysis of A still follows pseudo-first-order kinetics, because the second order reverse reaction is involved in the equilibrium.

$$\frac{d[A]}{dt} = -k_1[A] + k_{-1}[B][C] \quad \textbf{(3.3)}$$

According to Larsen *et al.*[144], in such cases, the pseudo-first-order rate constant (k_1) of the direct reaction can be estimated based on the initial and equilibrium concentrations of the species involved by:

$$\ln\left(\frac{a_0 b_{eq} + b a_{eq}}{a_0(b_{eq} - b)}\right) = \frac{2a_0 - b_{eq}}{b_{eq}} k_1 t \quad \textbf{(3.4)}$$

tSince the concentration of B at equilibrium (b_{eq}) and at time t (b) can be related to the original concentration of A (a_0) by $b_{eq}=a_0-a_{eq}$ and $b=a_0-a$, equation **3.4** can be rewritten strictly as a function of the concentration of A

$$\ln\left(\frac{a_0^{\,2} - a a_{eq}}{a_0(a - a_{eq})}\right) = \frac{a_0 + a_{eq}}{a_0 - a_{eq}} k_1 t \quad \textbf{(3.5)}$$

The graphical representation of this equation should provide a straight line for times preceeding equilibrium. The pseudo-first-order rate constant can be derived from the slope of that line from:

$$k_1 = slope \frac{a_0 - a_{eq}}{a_0 + a_{eq}} \quad \textbf{(3.6)}$$

The decay plots of each compound at different pHs were evaluated and assigned to one of these assumptions of degradation; *i.e.* to completion or to an equilibrium situation. Generally it was found that, at lower pH, elimination proceeded to completion whereas at neutral to basic pH an equilibrium was established. This was probably due to the protonation of the free amine in acidic conditions limiting the reverse reaction.

3.4.2. Test of compounds C01, C02, C03, C04, C05, and C06

All of the indanone derivatives from the original project were tested/retested under the new conditions. Aliquots of 50 µl of stock solutions in acetonitrile were diluted to 2 ml of each pre-warmed buffer at 37°C. Compounds **C03** and **C06** were in the hydrochloride salt form. Initial concentrations of the different compounds were as in Table 3.1.

Table 3.1: Initial concentrations of each compound in the buffered solutions

Compound	Concentration (mM)
C01	0.09
C02	0.07
C03	0.05
C04	0.09
C05	0.10
C06	0.19

The pseudo-first-order degradation rates were determined based on equation **3.2**.

Compound **C04** was one of the exceptional cases where the assumption of pseudo-first - order kinetics did not always provide the best representation of the decay in the concentration of the compound. At pH above five, the hydrolysis reaction apparently stopped before the compound was entirely consumed (Figure 3.6).

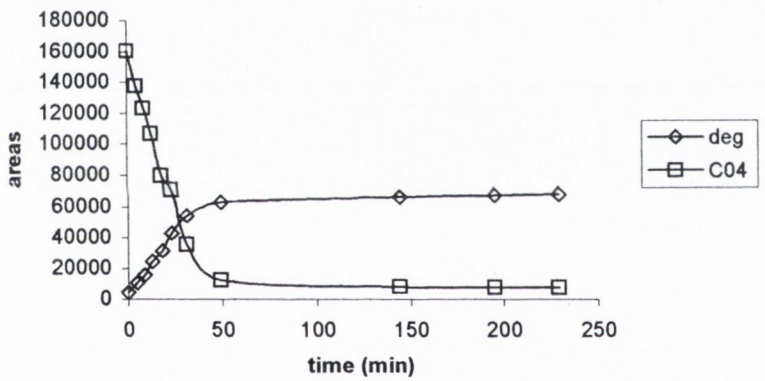


Figure 3.6: Areas against time of C04 (0.27 mM) and degradation product at pH=7.8

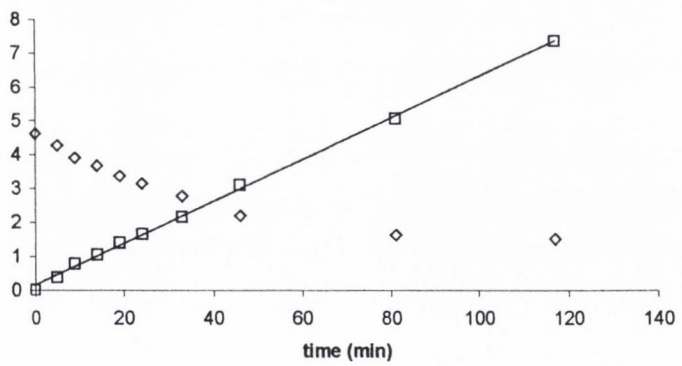


Figure 3.7: Plots of the ln of the remaining percentage of compound C04 (◊) (a_0= 0.09mM) and of the quantity $((a_0^2-aa_{eq})/(a_0(a-a_{eq})))$ (□)

In this case, the plot of the natural logarithm of the remaining percentage against time did not afford a straight line but the graphical representation based on equation **3.4** did, as illustrated in Figure 3.7, supporting the equilibrium theory.

The equilibrium theory was initially disregarded because, despite the fact that the concentration of **C04** seemed to stabilise, the areas of the degradation product still seemed to increase slightly. However, this was later rationalised with the slow degradation of 2-inden-1-one in solution. In this situation, the equilibrium is destabilised and the elimination reaction is driven forward, although slowly (Figure 3.8, $k_1 > k_{-1} >> k_2$).

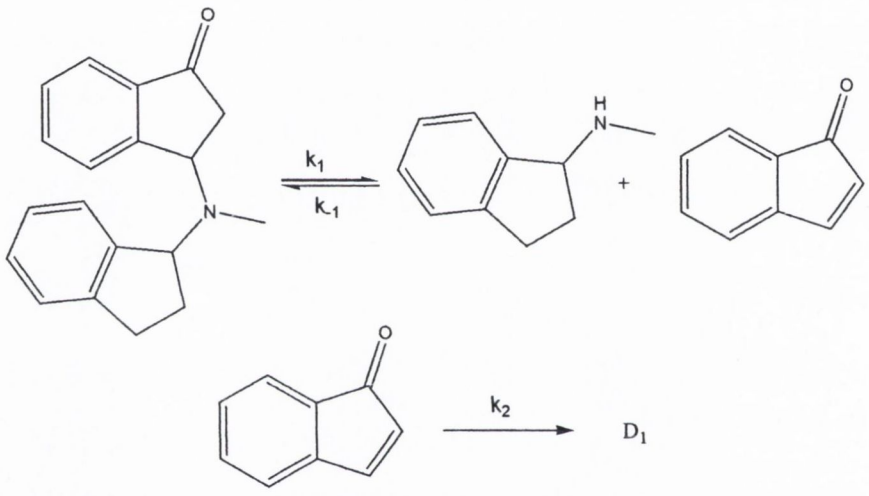


Figure 3.8: Destabilisation of the equilibrium of the elimination reaction of compound C04 by degradation of indenone

The rate constants obtained with equation **3.4** were not very different from the ones obtained by application of equation **3.2** but the coefficients of correlation were generally closer to one, if the equilibrium was considered at pH above five. An average difference of 7% (with a maximum of 28%) was found between the two determinations.

A test was done for degradation at different concentrations and pH. Results are presented in Table 3.2. Rates of the forward reaction seem to be faster at lower concentration (for basic pH), which is consistent with a smaller number of collisions between the neutral amine and the unsaturated ketone with consequent lower rates of the reverse reaction. The differences are statistically significant when compared with a repeatability test at a single concentration (n=5, RSD=14%).

Table 3.2: Degradation rate constants (min^{-1}) of C04 at different pHs and concentrations

C04 (mM)	2.7	5.1	7.8	10.5
0.05	0.00009	0.0149	0.100	0.076
0.09	0.00014	0.0126	0. 057	0.024
0.18	0.00010	0.0166	0.062	0.033
0.27	0.00019	0.0159	0.061	0.008

Although the other compounds of the group of derivatives of aminoindanes and cyclopentylamine were generally degraded to completion, there were a few cases, mainly at high pH, were the elimination profiles were better described by the equilibrium assumption. Therefore, the pH/rate constant profiles were constructed based on the theory that better described each particular case. Figure 3.9 depicts the pH/rate profiles for this group of compounds.

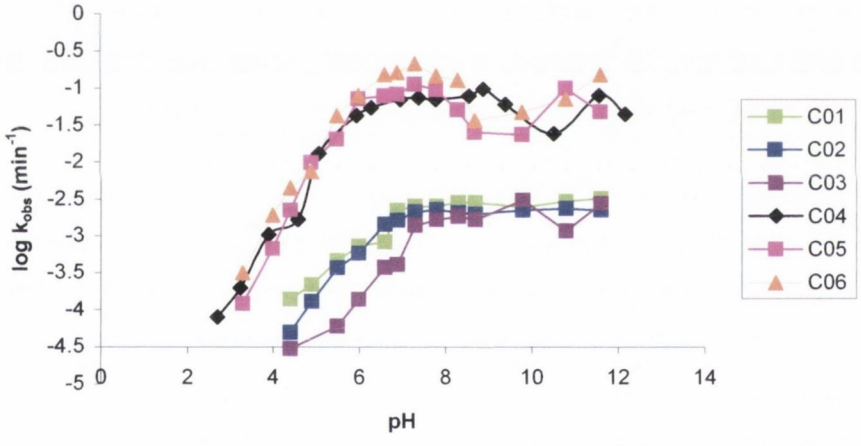


Figure 3.9: pH/rate profiles of C01, C02, C03, C04, C05, and C06, at 37°C and I=0.154

In general, the tertiary amines degraded faster than the secondary ones and at lower pH.

At pH between 0.5 and 2 there is some evidence of degradation of the tertiary amines as the peaks for the free amines can be detected. However the reaction is too slow to allow accurate determination of the rate constant by CE. In fact, the original concentration of the compounds appears to increase during the course of the experiments, which is possibly due to evaporation of the buffer.

The half-lives at pH=3.3 and 7.4 are presented in Table 3.3, illustrating the significant difference in the stability of the compounds in acidic and basic conditions.

Table 3.3: Half-lives at pH=7.4 and pH=3.3 (min)

Compound	pH=3.3	pH=7.4
C01	6931	272
C02	3850	324
C03	36657	495
C04	3465	9.4
C05	5728	6.2
C06	2166	3.2

As expected, in the cases of compounds **C06** and **C03**, no new peaks were observed during degradation, as the sensitivity for the detection of aliphatic amines is low with UV/vis. detection.

With the other compounds new peaks were observed which, in the case of **C01** and **C02**, had the same migration times (CE) and retention times (HPLC) as 1-aminoindane and 2-aminoindane respectively. The peaks of **C05** and **C04** were not identified but they were thought to be N-methyl-2-aminoindane and N-methyl-1-aminoindane respectively. Due to the characteristics of the CE method used, 2-inden-1-one was not detected, but it was identified by the HPLC retention time of a sample of the compound.

Quantification performed by HPLC revealed that the second eluting diastereomer of compound **C01** degrades approximately two-fold faster than the first one (0.0035 against 0.0018 min^{-1}) at pH 7.4. The average degradation rate was 0.0029 min^{-1}. After degradation of 75% of the original product in solution, 72% of the amount of 1-aminoindane expected after complete elimination had been recovered. This corresponds to a 96% recovery. The same procedure revealed a 94% conversion of compound **C02** to 2-aminoindane.

3.4.3. 3-Dimethylamino-indan-1-one (C12)

Buffered solutions of 50 mg/l (0.28 mM) of the product of the reaction of dimethylamine with 3-bromoindanone (**C12**) were used for the determination of the pH/rate profile of this compound in the pH range 3-11 (Figure 3.11).

At pH above five, there was a rapid initial decay in the concentration of the test compound followed by an apparent reduction in the rate of reaction (Figure 3.10).

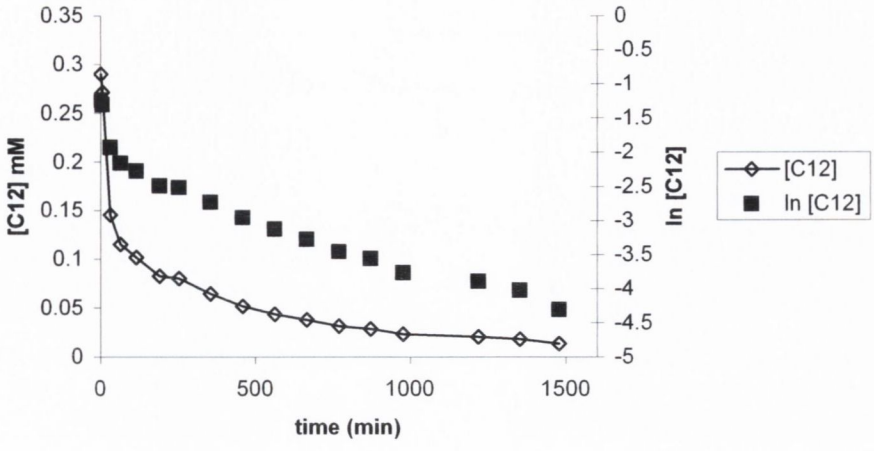


Figure 3.10: Decay in the concentration of C12 at pH=7.8

Recalculation of the rates based on equation **3.4** afforded a better correlation but there were still deviations from linearity at initial concentrations and this could be due to an erroneous attribution of the equilibrium concentration. Although a decrease was observed in the rate of the reaction after 30-60 minutes, there was no subsequent apparent plateau in the concentration of the original compound as was seen for **C04.** This means that equilibrium is possibly not the only phenomenon responsible for the deviation from first-order kinetics.

In this case, the subsequent degradation of 2-inden-1-one may have a more important role than in the case of the previously mentioned case of compound **C04**. This would happen if the rate of degradation of 2-inden-1-one were comparable to the rate of the reverse reaction of elimination.

Another possibility is that the compound is not completely soluble at the initial concentrations and the initial decay is the result, not only of degradation, but also precipitation. This steady reduction in the concentration of **C12** is consistent with the possibility that re-dissolution is limiting the rate of degradation. Basically this means that any determination of the profiles using the first portion of the curves may reflect mainly falling out from solution, while determinations based on the second portion may be strongly underestimated due to the slow returning to solution. This hypothesis could not be confirmed by determination of the solubility of the compound due to degradation.

However, since determination of the rate of hydrolysis based on the equilibrium assumption, provided values between these over and under estimations, it was considered a good estimate and was used for the construction of the pH/rate profile depicted in Figure 3.11.

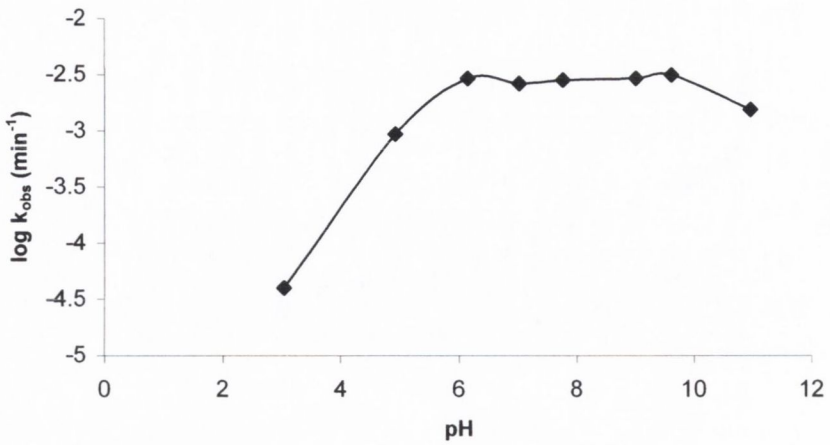


Figure 3.11: pH/rate profile of C12

Tests were carried over by HPLC at pH=0.5 revealing that the compound was stable at this pH. At pH=7.8 the half-life was 266 min.

3.4.4. 3-Ethylamino-indan-1-one (C13)

The reaction of ethylamine with 3-bromoindanone afforded two products. One of them was identified as **C13** and appeared to be pure.

With respect to the second compound, a second addition of an unsaturated ketone to the original product may have occured[209] (Figure 3.12).

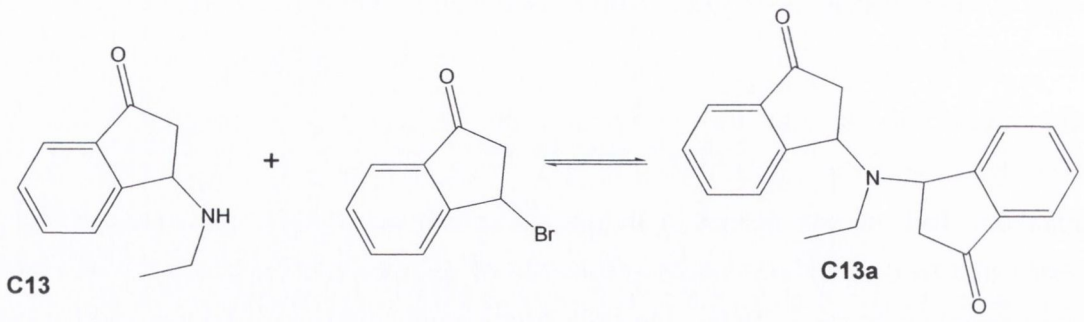


Figure 3.12: Formation of the di-indanone derivative of ethylamine

The pH/rate profile of compound **C13** was not determined. However, HPLC tests showed that the compound was stable at pH=0.5 and had a profile of degradation at pH=7.4 consistent with the establishment of an equilibrium with K=0.11. The first order degradation rate at this pH was 0.0008 min^{-1} ($t_{1/2}$=836 min).

3.4.5. 3-Piperidino-indan-1-one (C14) and 3-Piperidine-2-methylindan-1-one (C22)

Solutions of 102 mg/l (0.48 mM) of **C14** were tested in the range of pH of 2.8 to 11.7. The compound did not degrade below pH=4. However, the usual concentration effect, due to evaporation, occurred and this could have affected the observations. At pH=5 the concentration effect seems to be compensated by degradation and it looks like the concentration of the compound is not changing.

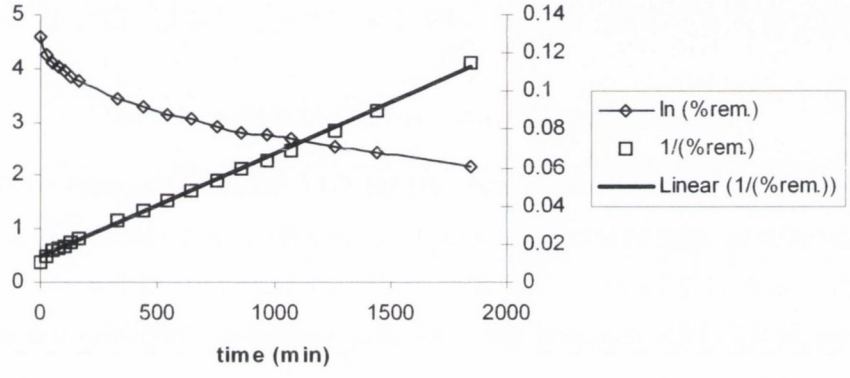


Figure 3.13: Evaluation of the order of the degradation reaction of compound C14

Above pH=6, decay profile is not well described by pseudo-first-order kinetics. Also it is difficult to assign an equilibrium due to the fact that a plateau in concentration was not detected. In this case, the decay seems to be better described by second order kinetics since the plot of the reverse of the remaining concentration against time provides a straight line (Figure 3.13).

This effect was observed mainly at pH between 7 and 10. At lower and higher pH, the reaction seems to be well described by pseudo-first-order kinetics. For this reason, the pH/rate profile of this compound was drawn only for pHs under seven (Figure 3.14).
No evidence of degradation was found in tests by HPLC at pH=0.5 and 3.8.

Compound **C22** was prepared by reaction between 3-bromo-2-methyl indanone and piperidine and the rates of disappearance of the compound from buffered solutions were studied. The initial concentration was 0.22 mM.

The rates of degradation of this compound are of the same order of magnitude as the degradation rates of compound **C14**. In this case the effect previously described for **C14** was not observed, as the disappearance of compound **C22** follows apparent pseudo-first-order kinetics at all pH values studied.

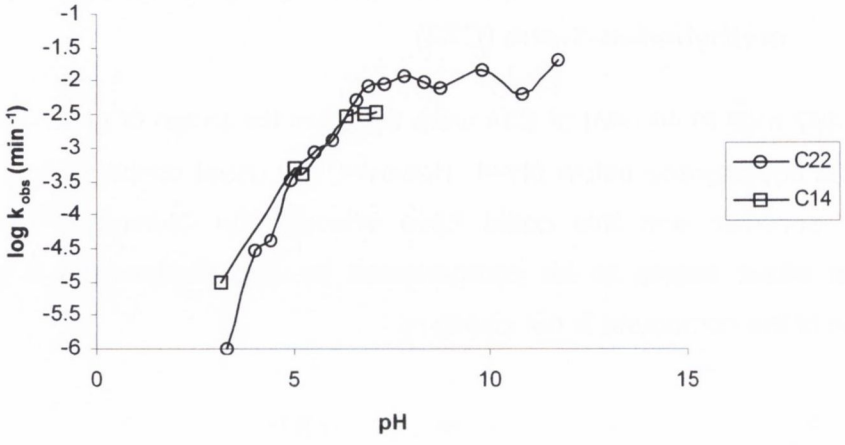


Figure 3.14: pH/rate profiles of C22 and C14

The apparent second order kinetics observed for **C14** could be caused by the secondary reaction involved or by degradation of 2-inden-1-one. HPLC analysis showed a reduction in the area of the peak of 2-inden-1-one after the initial increase. At the same time another peak appeared at RT=17.2 min and this was observed when following the degradation of all compounds. However there was also a new peak appearing at RT=14.9 during degradation of **C14**. Figure 3.15 illustrates the decay in the concentration of **C14** and concomitant formation and degradation of 2-inden-1-one as well as of new products.

Several factors appear to be influencing the determination of the rate constants for this compound, and equilibrium concentrations could not be determined. However, the possibility of reverse reaction was demonstrated by mixing 2-inden-1-one with a large excess of piperidine in buffer at pH=7.4. In this situation, all 2-inden-1-one was consumed and a peak was detected by HPLC at the retention time of **C14**.

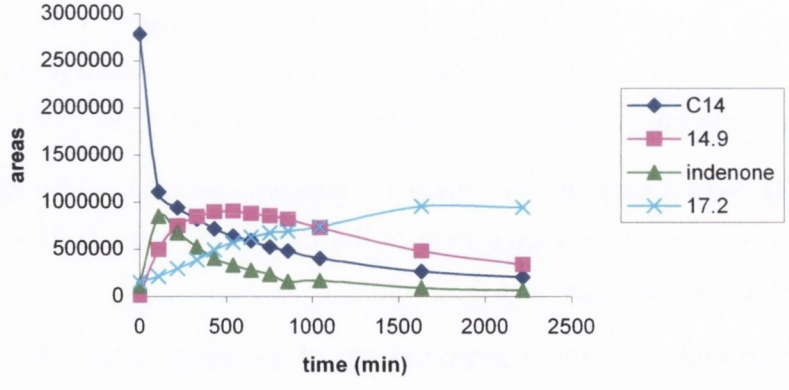


Figure 3.15: Profile of degradation of C14 and corresponding products of degradation (pH=7.4)

The effect of the ionic strength (0.05-0.5) of the buffer in the rates of degradation at pH=7.4 was also evaluated. Apparent second order kinetics was observed in every case providing some evidence that the effect was not due to low buffer capacity.

Rates of degradation were also determined using different concentrations of **C14** (0.45, 0.9, 1.8 ant 3 mM), by capillary electrophoresis and by HPLC. Apparent second order reaction was observed at all concentrations. Second order reaction rates obtained by both methods were in good agreement and are summarised in Table 3.4.

The fact that the second order reaction rate increases with the decrease in concentration of **C14**, reveals that the reaction is not truly second order and constitutes evidence that more than one reaction may be involved or that there is contribution of the second order reverse reaction to the overall kinetics.

Table 3.4: Second order degradation rates and half-lives of C14 at different concentrations determined by CE and HPLC

	CE		HPLC	
Concentration (mM)	K_{obs} ($M^{-1}min^{-1}$)	$t_{1/2}$ (min)	K_{obs} ($M^{-1}min^{-1}$)	$t_{1/2}$ (min)
0.45	n.d.	n.d.	29.13	76
0.90	6.05	184	9.34	119
1.8	3.38	164	4.83	115
3	1.99	167	2.44	137

3.4.6. 3-Phenethylamino-indan-1-one (C15)

Compound **C15** was prepared as a potential prodrug for 2-phenylethylamine. The compound was found to be unstable in solid state as confirmed by NMR analysis.

Elimination rates were determined by CE from solutions containing 1.06 mM of **C15** in the pH range 2.8 to 11.5. No decay in concentration was observed at pH under 5. However, an increase in the concentration of phenylethylamine was detected, which provided evidence of some slow elimination at low pH. Rates were calculated after compensation for evaporation.

The compound was found to degrade slowly at low pH, with a half-life of 6.9 days at pH 2.8. At basic pHs, the elimination rates are considerably faster with half-lives ranging from 3 to five hours at pH above 7. The pH/rate constant profile is represented in Figure 3.16.

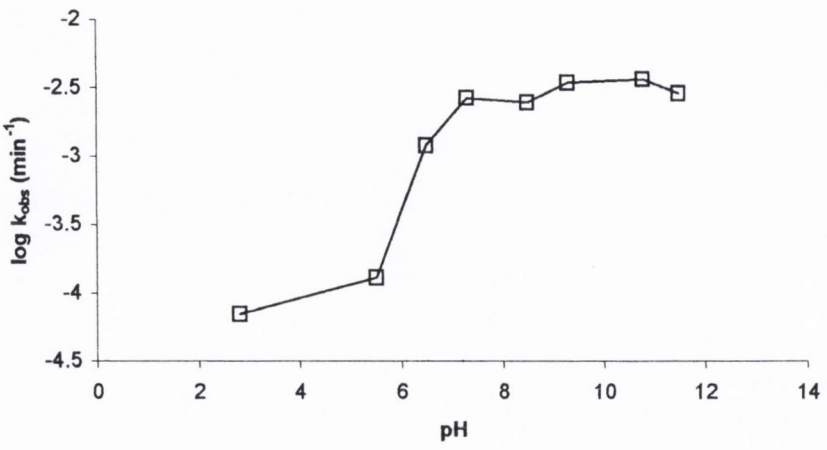


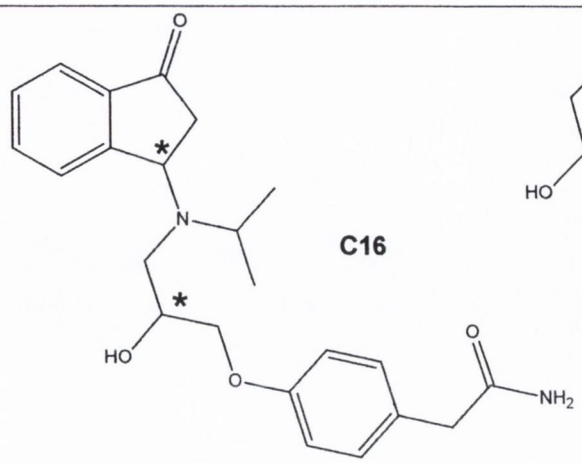
Figure 3.16: pH/rate profile for compound C15

3.4.7. 3-Atenolol-indan-1-one (C16)

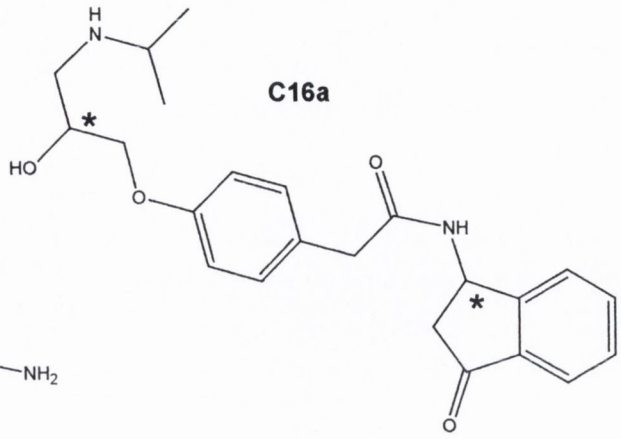
Atenolol is a β-adrenoceptor blocking agent that, like other drugs of this class, is used in the treatment of high blood pressure and in the prophylaxis of anginal attacks[192]. Compared to some β-adrenoceptor blocking agents (like propranolol), which may also affect other β-adrenoceptors including the peripheral smooth vasculature and the bronchi, atenolol is more specific for the heart β-adrenoceptor. Unlike other initially promising drugs like dichloroisoproterenol (DCI), atenolol also does not show intrinsic sympathomimetic activity[192].

In the context of this work, the drug was used as model as there is no important justification for the production of a prodrug. However, prodrugs of the related propranolol have been extensively investigated.

Two products could be expected from the reaction of atenolol with 3-bromoindanone, resulting from the substitution of bromine by the amine (**C16**) or the amide (**C16a**) functions of atenolol. In both cases, pairs of diastereomers would be obtained as atenolol already carries one chiral centre.



C16



C16a

If substitution by the amide occurred, a different shift would be observed for the CH_2 group next to this function in the NMR spectra of the addition product when compared with atenolol. On the other hand, the CH_3 protons from the isopropyl group as well as the protons of the CH and CH_2 groups close to the amine function should remain in the same position. However, the opposite was observed revealing substitution by the amine. The two chiral centres of the product of the reaction (**C16**) account for the two spots observed by TLC. The two spots were separated by flash chromatography although only the firstly eluting diastereomer was obtained uncontaminated from the other and this compound was used for kinetic tests.

a) Kinetic tests by spectrophotometry

Spectrophotometry was tentatively used to determine the rate of elimination of **C16** by following the increase in the concentration of 2-inden-1-one. Figure 3.17 displays the UV/visa spectra of 2-inden-1-one in a aqueous buffer at pH=7.4. Some interference may be present, at low wavelength, due to acetonitrile present in the stock solution but the maximum of absorbance at 323 nm is clearly visible.

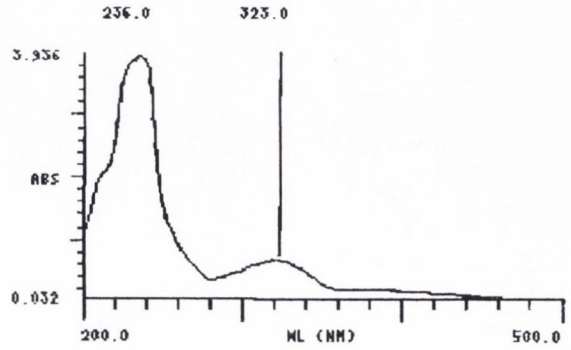


Figure 3.17: UV/vis spectrum of 2-inden-1-one in aqueous solution at pH 7.4

Since atenolol has a maximum of absorbance at 275 nm, there was also the possibility of following its formation by determining the absorbance at this wavelength. However, since **C16** also absorbs in this region, that is not possible.

Solutions of **C16** were placed in a quartz cuvette in the cell compartment of an UV/vis spectrophotometer and spectra were registered at regular intervals and an increase in the absorbance was evident at 323 nm (Figure 3.18). However, the method was later found to be unsuitable due to the degradation of 2-inden-1-one in solution.

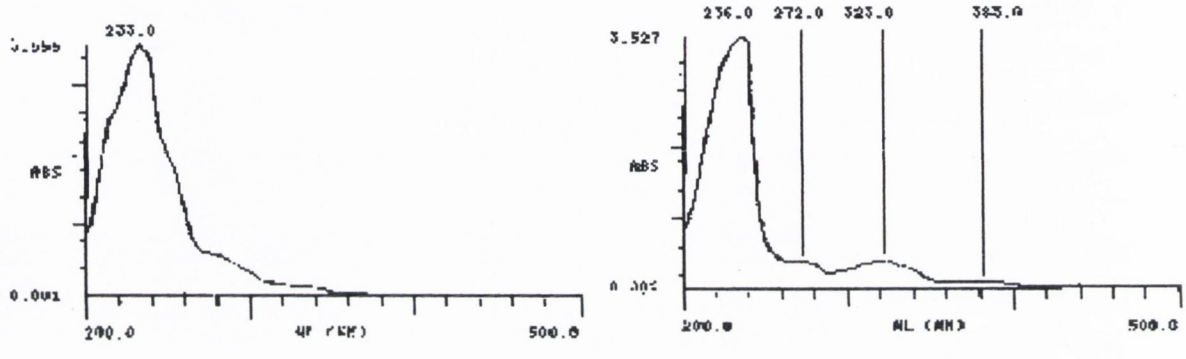


Figure 3.18: UV/vis spectrum of C16 (0.35 mM) in aqueous solution at pH 7.4 before and after degradation

b) Kinetic tests by CE

For the kinetic tests, a 100 mg/l solution of the purest diastereomer was used and only one peak was seen by CE analysis. The second fraction separated, also accounted for only one peak by CE at the same migration time.

Concurrently with the decrease in the concentration of the original compound, another peak appeared in the electrophoregram. The migration time of this peak coincided with the migration time of atenolol.

The reaction is pseudo-first-order (coeff. corr.>0.95) in the range of pH tested and no evidence of equilibrium was found. The profile of the first-order reaction rate coefficient against the pH is plotted in Figure 3.19. The half-life of the product is 192 min at pH=3.04 and less than 5 min for pH above 6.

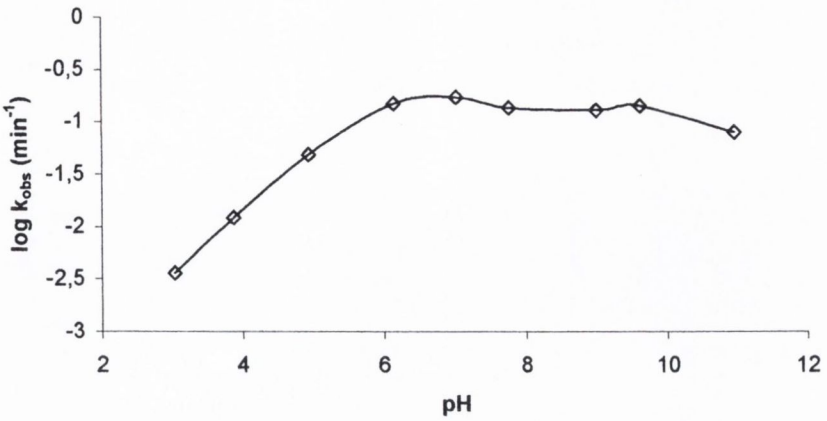


Figure 3.19: pH/rate constant profile of C16

A test, at pH=3.04 and pH=4.93, of the fraction resultant from the second spot seen by TLC, showed similar rates of degradation.

c) Kinetic tests by HPLC

Since the rates of hydrolysis of this compound are so high, and HPLC runs are much longer than CE runs, samples had to be quenched to stop the reaction before analysis by HPLC.

Solutions of **C16** in buffers at pH=0.5, 3.9, 7.1 and 7.4 were prepared and kept at 37°C. Aliquots (0.2 ml) were taken at appropriate time intervals, quenched with 0.3 ml of a 5% (v/v) perchloric acid solution and analysed by HPLC. A repeatability test was done at pH=7.4 (n=3, k_{obs}=0.545±0.016).

Retention times for atenolol, **C16** and 2-inden-1-one were 4.0, 10.8 and 15.1 respectively. 2-Inden-1-one could not be quantified since the areas of its peaks where dependent on the time before injection; the longer the samples were kept before injection, the smaller the peak would be and another peak would appear at 8.8 min. This peak is thought to be a degradation product of 2-inden-1-one forming only in quenched solutions. The peaks of atenolol and **C16** were repeatable upon injection of the same sample at different times.

Figure 3.20 represents the pseudo-first-order curves for the disappearance of **C16** in various solutions and illustrates the significant difference in stability in acidic and neutral conditions as well as the excellent agreement with pseudo-first-order kinetics.

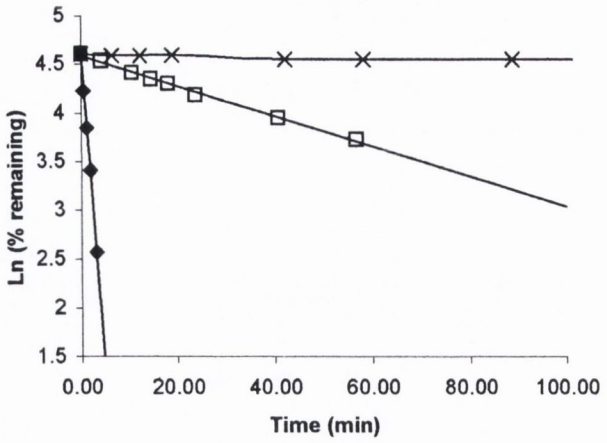


Figure 3.20: Pseudo-first-order curves for the disappearance of C16 in various solutions at 37°C: pH 7.1 (-♦-), pH 3.8 (-□-), pH 0.5 (-x-)

The rates of hydrolysis of **C16** and respective half-lives are presented in Table 3.5.

Table 3.5: Elimination rates and half-lives of C16 determined by HPLC

pH	K_{obs} (min^{-1})	$t_{1/2}$ (min)
0.5	0.0002	3467
3.9	0.010	67.3
7.1	0.685	1.0
7.4	0.545±0.016	1.27±0.04

3.4.8. 3-(Tryptophan methyl ester)-indan-1-one (C17)

The β-aminoindanone derivative of tryptophan methyl ester (**C17**) was tested in the pH range of 3.04-9 in solutions containing 140 mg/l of the compound. In this case there are two peaks appearing in the electrophoregrams due to the two pairs of diastereomers of the compound. As it will be seen later, the pK_a of the compounds is close to the pH of the running buffer which may account for slight differences in the degrees of ionisation, which may be responsible for the different migration times and consequent resolution of the diastereomers. The two compounds were not resolved by HPLC.

The compounds seem to degrade over the pH range tested, the degradation being faster at pH=5, with a half-life of 277 min. There are only small differences in the degradation rates of the two diastereomers (Figure 3.21). At the same time that depletion is observed in the

peak areas of these compounds, a new peak appears at the migration time of tryptophan methyl ester.

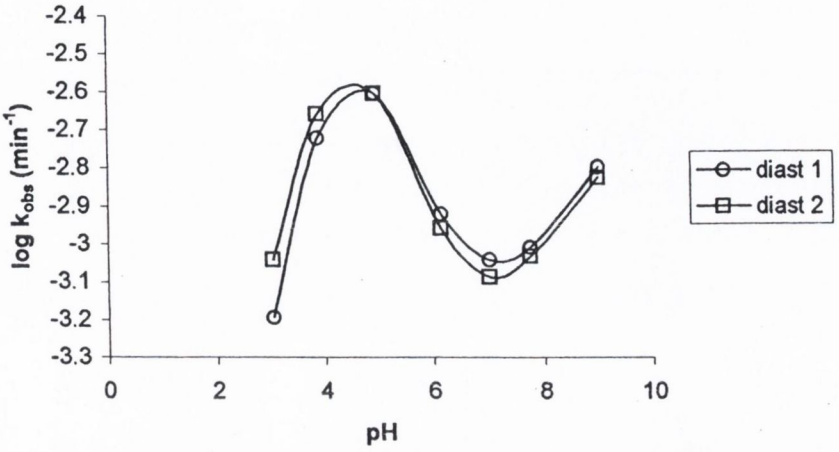


Figure 3.21: pH/rate profiles for the two diastereomers of C17

At pH=4, tryptophan methyl ester and 2-inden-1-one seem to be the only products of degradation of **C17**. However, at higher pH this compound seems to hydrolyse further to tryptophan as confirmed by similarity of retention times by HPLC (Figure 3.22). Nevertheless, deamination seems to be faster than ester hydrolysis since no other peak appeared that could be attributed to tryptophan-indanone.

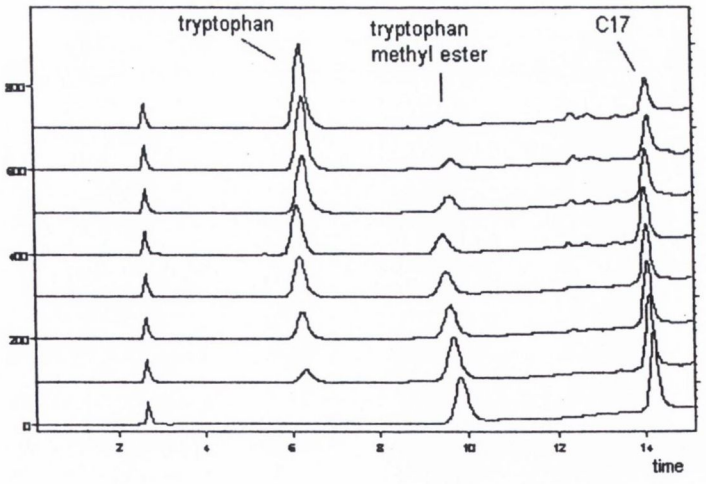


Figure 3.22: Degradation of C17 to tryptophan methyl ester and tryptophan at pH 7.4 (increasing time from bottom to top)

3.4.9. 3-(Alanine methyl ester)-indan-1-one (C18)

The product of the reaction of alanine methyl ester and 3-bromoindanone (**C18**) is constituted by a pair of diastereomers and produces two adjoining peaks by capillary electrophoresis. The two compounds were not separated previous to analysis.

Solutions of the racemic mixture (100 mg/l, 0.43 mM) were tested by capillary electrophoresis in the pH range 3.04 to 10.96. The observed degradation rates were calculated by the decay in the total area for the two diastereomers and are plotted in Figure 3.23. Degradation is faster at pH=6.14 where the half-life is 231 min. There was no observable degradation at pH=3.04.

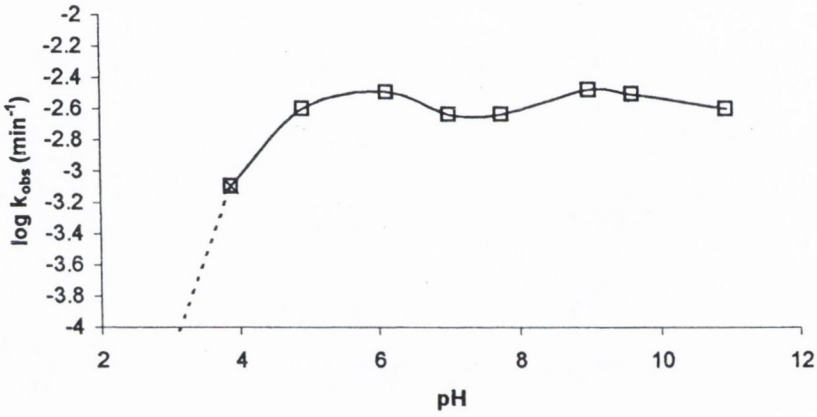


Figure 3.23: pH/rate profile of C18

Although it was impossible to accurately calculate the degradation rates for the two diastereomers separately, there is some evidence (from the relative heights of the peaks) that the second eluting diastereomer degrades faster than the first one.

3.4.10. 3-(Phenylalanine benzyl ester)-indan-1-one (C19)

The test of the kinetics of degradation of the compound (**C19**) was not done due to the systematic blockage of the capillary upon injection (CE), which was probably due to low solubility of the compound in the running buffer.

3.4.11. 3-Desloratadine-indan-1-one (C20) and 3-Desloratadine-2-methyl-indan-1-one (C23)

Loratadine is a carbamate derivative of azatidine and is an innovative drug in the sense that it was one the first non-sedating antihistamines. The reason why loratadine is non-sedating appears to be related to its low CNS penetrability despite being highly lipophilic[193]. Although it was not noticed immediately, loratadine is in fact a prodrug since the carbamate

group is displaced enzymatically to release an amine, desloratadine, which is the active drug.

The reaction of desloratadine with 3-bromoindanone afforded the target product (**C20**). Solutions of 144 mg/l (0.33 mM) of the product in buffer were tested.

The apparent rates of elimination were calculated based on equation **3.2** for pH<6 and based on equation **3.4** for pH>6 where there was strong evidence of establishment of an equilibrium (Figure 3.24).

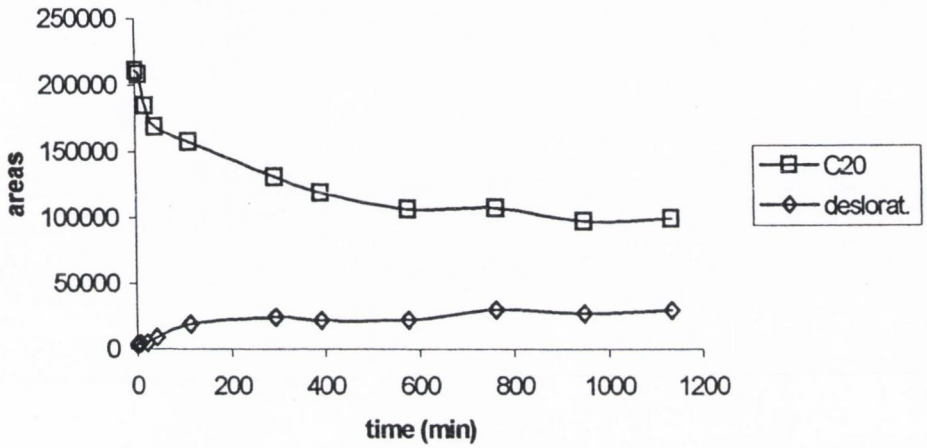


Figure 3.24: Degradation profile of C20 at pH=7.9

The compound resulting from the reaction of desloratadine with 3-bromo-2-methyl indanone (**C23**) degraded generally at slower rates than compound **C20** (Figure 3.25).

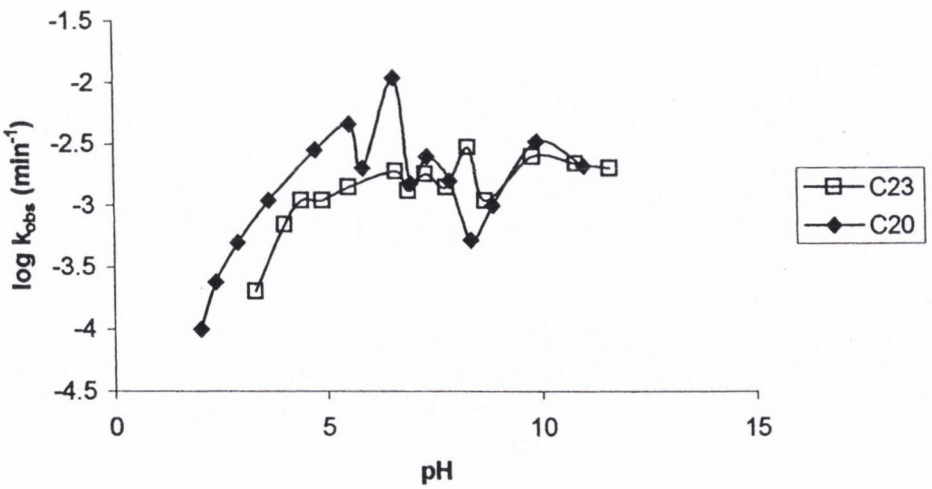


Figure 3.25: pH/rate profiles of C20 and C23

In this case, equilibrium was evident at pH values above 5.5.

The compounds are stable at pH<2. At pH=2.3 **C20** degrades but its half-life is greater than two days. Irregularities in the curves at high pH reflect difficulties in establishing the equilibrium concentrations. Some solubility problems may also exist particularly at around pH=6.5 where the decay in concentration was not always reflected in the production of desloratadine.

3.4.12. 3-Ephedrine-indan-1-one (C21)

C21 was obtained as a pair of diastereomers from the reaction of ephedrine with 3-bromoindanone. The two compounds were not separated and the NMR spectra described in Chapter 7 corresponds to the mixture. Non chiral capillary electrophoresis partially resolved the two diastereomers. The second eluting compound appears to degrade slower than the first one at some pH values as illustrated for pH=7.3, in Figure 3.26.

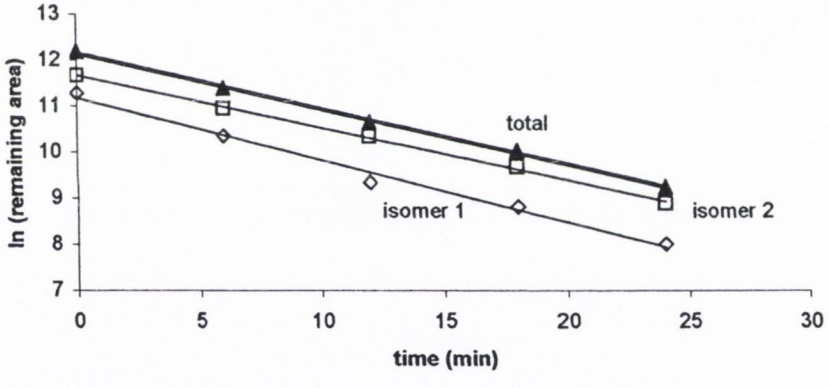


Figure 3.26: Pseudo-first-order degradation rates of ephedrine isomers (pH=7.3)

A pH/rate coefficient profile was determined in the range of 2.8 to 11.5, from the decay in the total concentration of the two diastereomers, starting with solutions containing 0.68 mM of the compound (Figure 3.27).

Elimination was found to occur at all pH values with maximum rates in the range of pH of 5.5 to 8.5 and half-lives of approximately 5 minutes. The apparent decay in the rate at pH between 9 and 10 may be due to low solubility or a change in the mechanism of reaction since pseudo-first-order kinetics did not adequately describe elimination observed at these pHs. Determination of the rates based on the theory of equilibrium did not afford better fits.

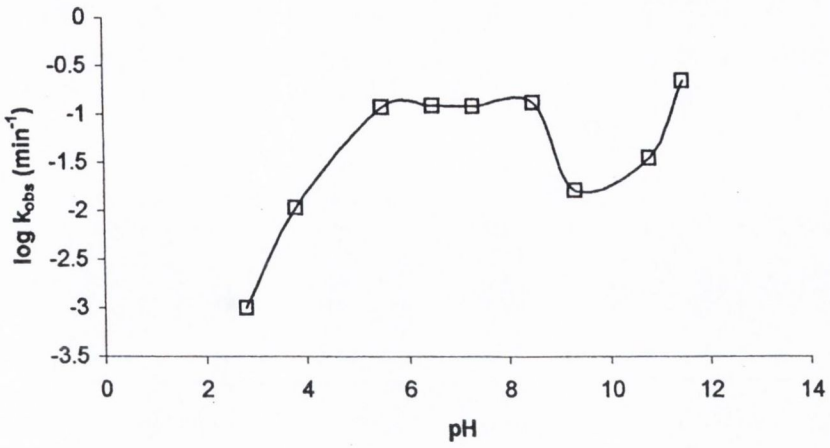


Figure 3.27: pH/rate profile for compound C21

3.5. Test of the degradation rates of aminoindanones in plasma

Some compounds were tested for hydrolysis in 100% human plasma.

Since plasma cannot be analysed directly in the capillary electrophoresis, incubation had to be done off line and samples had to be quenched and deproteinised before injection. In order to ensure there were no methodological effects on the determination, pH=7.4 buffer solutions were analysed simultaneously and in the same manner as the plasma samples.

Plasma and pH=7.4 buffer solutions of the test compounds were pre warmed at 37°C. Aliquots (0.2 ml) were taken at time intervals and quenched with 0.3 ml of a 5% v/v perchloric acid solution. Samples were centrifuged for 10 min at 3000 rpm and were then injected into the capillary electrophoresis.

No differences were expected in the degradation rates in plasma when compared to degradation in buffer at pH=7.4 and I=0.154, because the mechanism of degradation was thought to be strictly chemical.

Compound **C05** was tested at a concentration of 0.40 mM in plasma and buffer. Rates of elimination were 0.042 and 0.039 min^{-1} respectively. Compound **C06** was tested in a concentration of 0.75 mM. Rates in plasma and buffer were respectively 0.032 and 0.051 min^{-1}.

3-Atenolol-indan-1-one (**C16**) was tested for elimination of atenolol in plasma with a starting concentration of 0.16 mg/ml. Three samples were prepared by adding 30 μl of a stock solution containing 16 mg/ml of **C16** in acetonitrile to 3 ml of pre-warmed plasma and were kept at 37°C. 0.2 ml aliquots were taken periodically and were quenched with 0.3 ml of a

5% (v/v) perchloric acid solution after which they were analysed by HPLC. Figure 3.28 depicts three progress curves for the degradation of **C16** in plasma and corresponding quantitative elimination of atenolol.

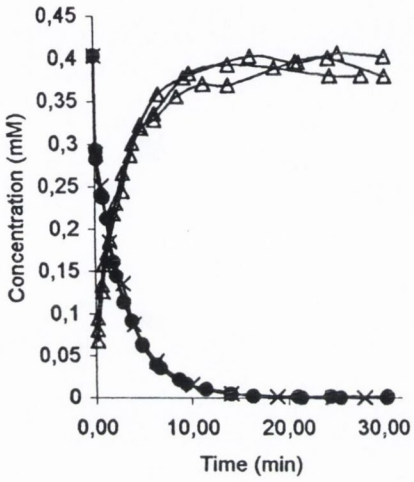


Figure 3.28: Progress curves for the disappearance of C16 (–•–) and the appearance of atenolol (–Δ–) in plasma

Buffer solutions at pH=7.4 were prepared in the same way for comparison. Results of the individual determinations are in Table 3.6.

Table 3.6: Results of the determination of the hydrolysis rate constants of C16 in plasma and buffer pH=7.4

	Buffer		Plasma	
	k_{obs} (min^{-1})	$t_{1/2}$ (min)	k_{obs} (min^{-1})	$t_{1/2}$ (min)
1	0.5561	1.25	0.2956	2.34
2	0.5294	1.31	0.2936	2.36
3	0.5498	1.26	0.3088	2.24
average	0.545±0.016	1.27±0.04	0.299±0.009	2.32±0.07

The rates of reaction were slightly smaller in plasma and this may be due to some protection afforded by protein binding. This also confirms that there is no important contribution of the plasma enzymes to the elimination reaction. Average recoveries of 94.0±4.4% and 99.2±1.9% (95% confidence) of atenolol were observed in buffer and plasma samples respectively.

3.6. Degradation of 2-inden-1-one and its effect on the kinetics of elimination

During the hydrolysis tests by CE, generally no other peaks were detected besides the β-aminoindanones and corresponding amines. A peak thought to correspond to 2-inden-1-one was detected when running buffers were used without TBA, but the spectra obtained with diode array detection did not always agree with the UV spectra of 2-inden-1-one. This was later rationalised by the degradation of 2-inden-1-one in solution that was confirmed by HPLC. The degradation products of 2-inden-1-one are likely to be uncharged molecules and not separated from 2-inden-1-one by CE as all uncharged molecules are expected to migrate as a single band under such conditions. By HPLC, peaks were detected as a result of degradation of 2-inden-1-one as mentioned before but they were not identified.

Degradation of 2-inden-1-one is probably the origin of the deviations to first-order kinetics in the presence of equilibrium. In this case, the overall system consists of the one depicted in Figure 3.29 where D_1 represents the first degradation product of indenone.

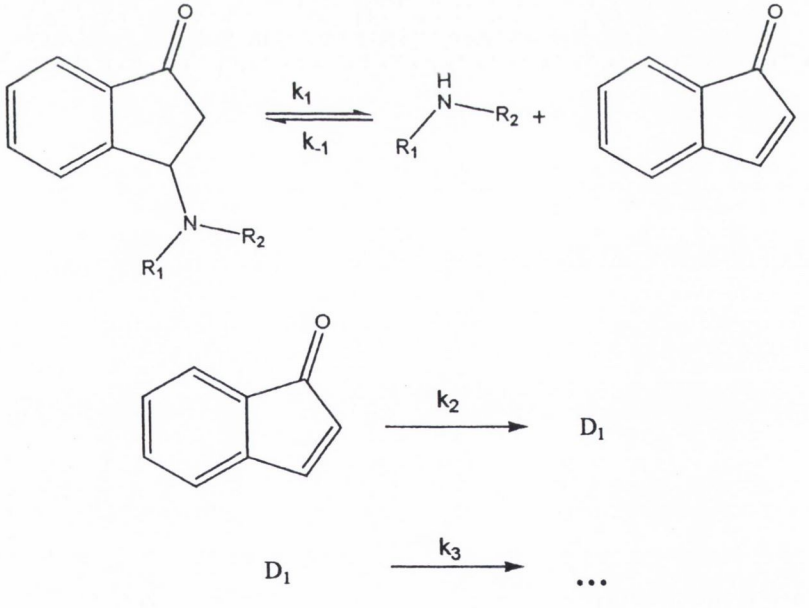


Figure 3.29: Role of degradation of indenone in the elimination system

In order to determine the first-order rate k_1 for this system, more information would be needed on the rates of degradation of indenone, however if $k_2 << k_{-1}$ the assumption that only the first reaction is occurring is admissible and the k_1 can still be obtained through equation **3.6**. On the other hand, if $k_2 >> k_1 >> k_{-1}$ (which means that indenone is consumed quickly after being produced), equation **3.2** should provide a fairly good estimate of k_1.

One of these situations must apply to most of the compounds; however, compounds such as **C12** and **C14** or **C21**, whose degradation was not well described by these equations may have $k_2 \approx k_{-1}$.

2-inden-1-one also degraded in solid state affording white crystals. The compound is known to dimerise photochemicaly[194,195] but the NMR of the compound obtained from indenone upon storage (**B15,** Chapter 7) did not agree with any of the published spectra.

3.7. Establishing a kinetic equation and a reaction mechanism

The pH/rate profiles for the β-aminoindanones generally have a sigmoid shape. This can be accounted for, by assuming spontaneous decomposition of the protonated and unionised form of the compound (Figure 3.30).

As seen in the previous sections, no specific acid catalysis was detected, as this would be revealed by a large increase in the rates of degradation at low pH[144,145]. Base catalysis is also not evident but it may have some contribution as will be shown later.

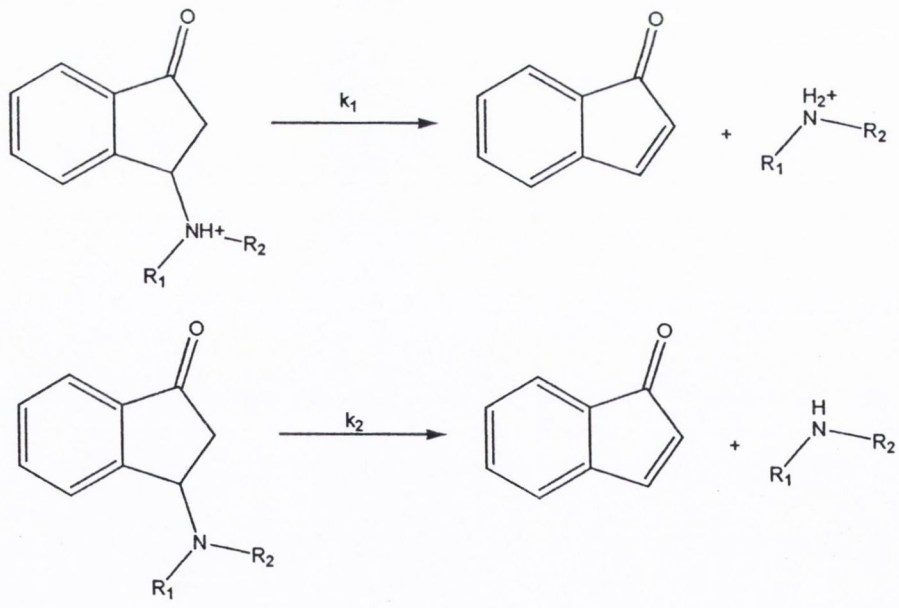


Figure 3.30: Individual contributions to the overall rate of elimination from indanone derivatives

The overall rate constant is dependent solely on the individual degradation rates of the ionised and unionised base and on the degree of ionisation at each particular pH:

$$k_{obs} = k_1 \frac{a_{H+}}{a_{H+} + K_a} + k_2 \frac{K_a}{a_{H+} + K_a} \qquad (3.7)$$

In this equation, k_1 and k_2 are the pseudo-first-order rate constants for spontaneous degradation of the protonated and unionised compound respectively. The terms $a_{H+}/(a_{H+}+K_a)$ and $K_a/(a_{H+}+K_a)$ represent respectively the protonated and free base fractions of the compound at each pH.

This expression can be rearranged to:

$$\log\left(\frac{k_{obs}-k_1}{k_2-k_{obs}}\right)=\log K_a-\log a_{H+} \qquad \textbf{(3.8)}$$

and can be reorganised in the form of a particular case of a Boltzmann sigmoid, which is

$$k_{obs}=k_2-\frac{k_2-k_1}{10^{(pH-pKa)}+1} \qquad \textbf{(3.9)}$$

Equation **3.9** can be fitted to the experimental points of the pH/rate coefficient profiles of each compound by non-linear regression.

This type of fitting affords the values of the bottom and top segments of the curve which represent k_1 and k_2 respectively, and also the pH at the inflexion point of the curve which corresponds to the pK_a.

Fittings were performed using GraphPad Prism[196] for all compounds, generally in the range comprised between the lower degradation rate observed at low pH, and the highest at pH under 8. Results above this pH were not used since in many cases a depression in the profiles was observed, which cannot be accounted for by this kinetics equation. Figure 3.31 illustrates the fitting curve provided by equation **3.9** for compound **C16**.

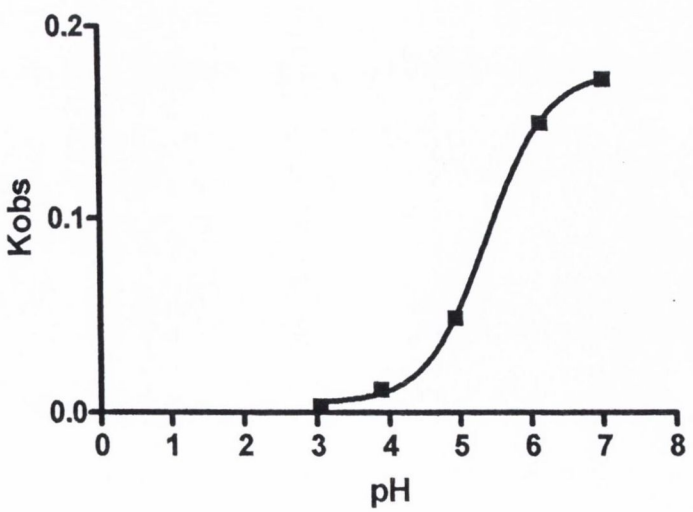


Figure 3.31: Fitting of equation 3.9 to the experimental data of C16

The values of k_1, k_2 and pK_a obtained through this procedure for the test compounds are listed in Table 3.7. Negative values were obtained for k_1 in some cases but they are smaller than the expected error of data inputted to non-linear regression.

Table 3.7: Parameters of the fitting of the pH/rate profile of the various compounds

compound	k_1 (min^{-1})	k_2 (min^{-1})	pK_a
C01	9e-5±0.0002	0.0029±0.0003	6.45±0.24
C02	8e-5±0.0001	0.0022±0.0001	6.31±0.16
C03	1e-5±0.0002	0.0024±0.0004	7.32±0.40
C04	-0.00022±0.0023	0.073±0.003	5.72±0.10
C05	-0.0010±0.011	0.10±0.01	5.77±0.35
C06	0.0018±0.0099	0.22±0.03	6.19±0.20
C12	-9e-5±0.0004	0.0029±0.0003	5.08±0.37
C14	1e-7±3e-5	0.0033±3e-5	5.93±0.03
C15	-3e-5±0.0006	0.0030±0.0.0007	6.55±0.61
C16	0.0049±0.0071	0.18±0.01	5.31±0.15
C17	-3e-5±0.001	0.0026±0.0011	3.27±0.95
C18	1e-7±0.0003	0.0032±0.0004	4.30±0.32
C20	0.0003±0.0005	0.0064±0.0010	4.87±0.35
C21	-0.0046±0.028	0.13±0.01	4.49±0.56
C22	-3e-5±0.0004	0.012±0.001	6.60±0.15
C23	3e-5±0.0005	0.0016±0.0004	4.14±0.67

As already stated, these sigmoid curves do not account for the pH/rate profile in the whole pH range as some profiles are represented by a bell shaped curve in the range of pH of 3-9. At higher pH, in such cases there is usually an increase again in the rate of degradation. It seems likely however that this is due to a significantly different mechanism.

Bell shaped curves can sometimes be accounted for in terms of two acid-basic dissociations of the substrate. They correspond essentially to two sigmoid curves back-to-back[197].One possible justification for the decrease in the rates at pH=8-9, which originates the bell shaped profile for the test compounds is therefore the acid-base equilibrium of the free base.

According to the kinetics equation, the reaction only happens if the aminoketone is in its neutral form. This introduces a limitation for the reaction at low pH (Figure 3.32). On the other hand, in order for the backwards reaction to happen, the free amine has to be unprotonated, so, as the pH increases, the rate of the reverse reaction increases too and consequently the observed rate is reduced.

At higher pH where both amines are unprotonated, there is also the possibility of some base catalysis increasing the overall rate of the direct reaction and overcoming the reverse reaction.

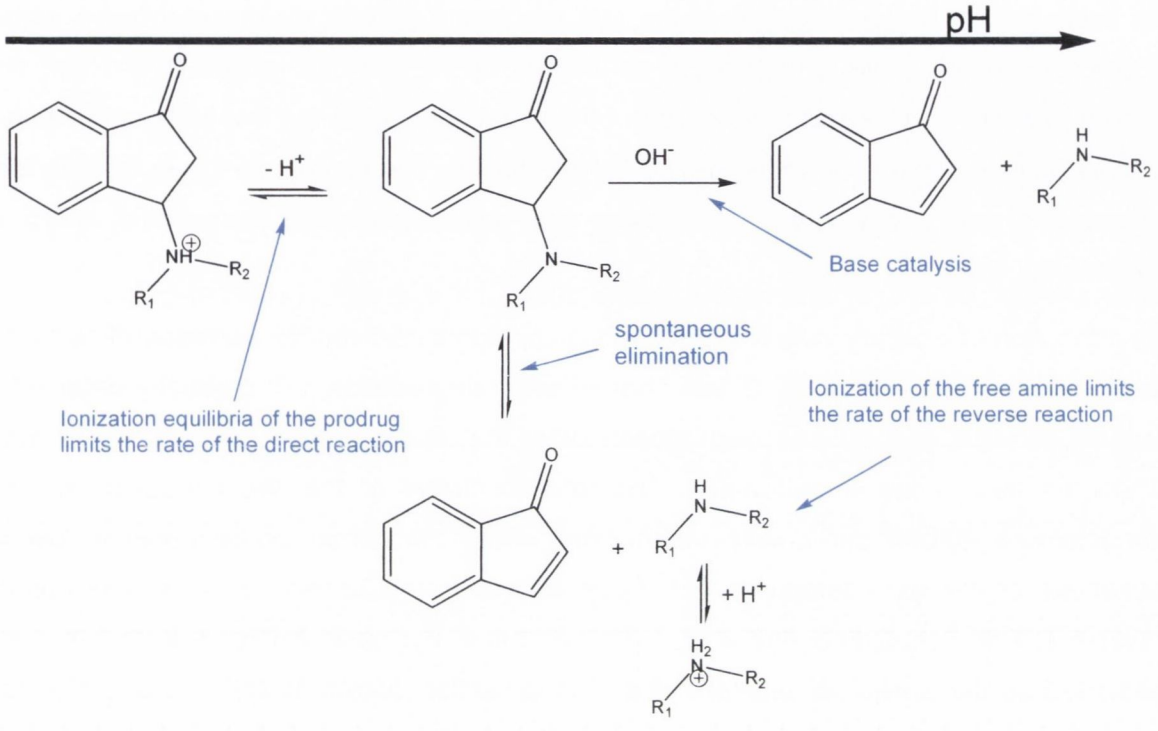


Figure 3.32: Factors influencing the rate of the elimination reaction at different pHs

Let fA^+ and fA^0 represent the prodrug in the protonated and neutral forms respectively while fB^+ and fB^0 represent the amine. The rate of the reaction (r) at a particular pH, calculated based in Figure 3.32, should be:

$$r = k_1[A^+] + k_2[A^0] - k_3[B^0][C] - k_4[B^+][C] + k_5[A][OH^-] \quad \textbf{(3.10)}$$

Consequently, the observed rate coefficient is dependent of the contribution of the acid-base equilibrium of the species involved:

$$k_{obs} = k_1 fA^+ + k_2 fA^0 - k'_3 fB^0 - k'_4 fB^+ + k'_5[OH^-] \quad \textbf{(3.11)}$$

Expression **3.11** is an approximation since pseudo-first-order kinetics were assumed for the second order reactions, but still the dependence on the pH should be similar.

According to non-linear fitting of the pH/rate profiles, k_1 and k_2 could be estimated, as seen before, but the contributions from the other reactions involved were left aside due to the complexity of the maths involved. Also, the relevance of the behaviour at high pH for the evaluation of the system as a prodrug system is small.

However, a simulation can be made to illustrate the similarity of a pH/rate profile obtained, taking into account all these contributions and the pH/rate profiles of some of the test compounds.

As seen before, the contribution of the first two terms affords a sigmoidal curve whose position in the pH scale is dependent on the pK_a of the prodrug. Intuitively one can also accept that terms three and four afford another sigmoidal curve but this time its position in the pH scale is dependent on the pK_a of the free amine. The contribution from the fifth term however is only dependent on the water acid-base equilibrium and should afford an exponential line.

By attribution of arbitrary values to each of the constants involved the corresponding overall profile can be determined. $k_1=0$ was chosen since degradation is not usually detected at low pH. A value of $k_3=0$ was also chosen since the reverse reaction is unlikely to occur when the free amine is protonated. The rate coefficient of the direct reaction in basic conditions, $k_2=0.025$ min^{-1}, was taken from within the range of deamination usually observed for the test compounds (c.f. Table 3.7) and $k_4=0.02$ min^{-1} was assumed as the reverse reaction should be slower than the direct one. For basic catalysis, $k_5=1$ min^{-1} was assumed as there was no estimate of it. The ionisation constants ($pK_a^A=5$ $pK_a^B=9$) were established from the general observations in Table 3.9. Based on these, the plots of the individual contributions of each acid basic equilibrium were drawn (Figure 3.33).

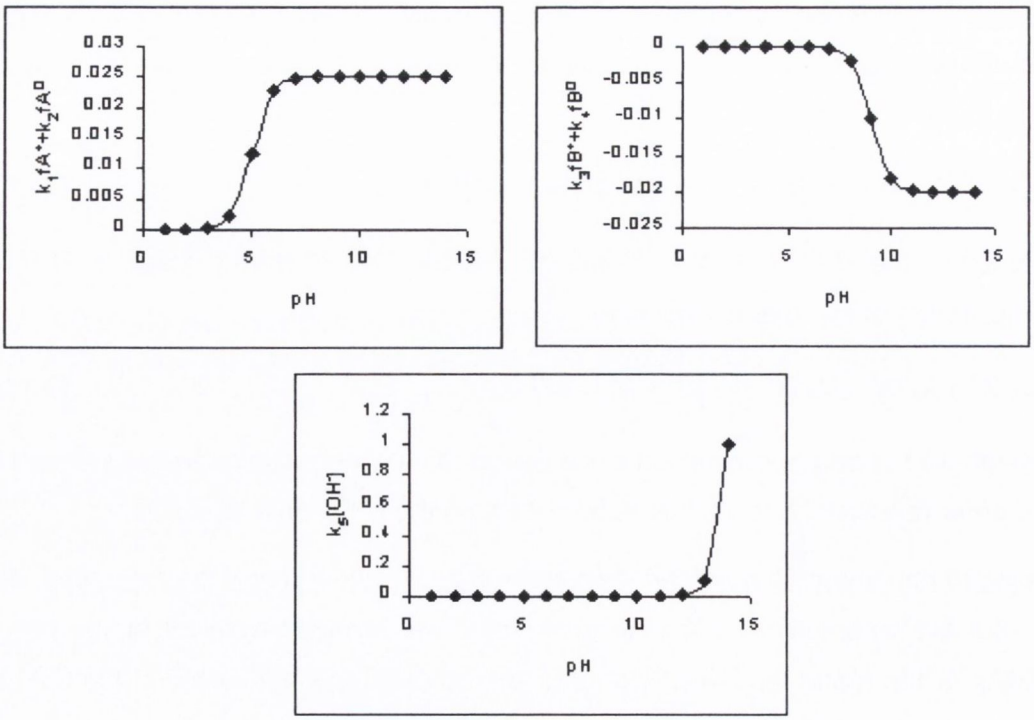


Figure 3.33: Individual contributions for the observed rate

The logarithm of the sum of the three curves affords a pH/rate profile depicted in Figure 3.34, where a dip is observed at pH above neutrality as noticed for some of the test compounds.

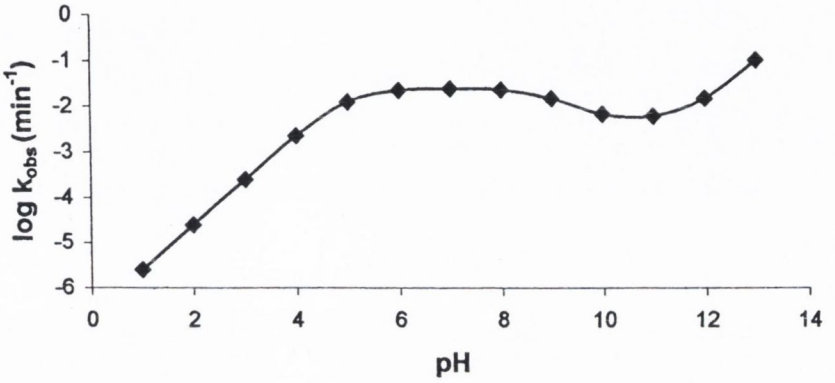


Figure 3.34: Simulated pH rate profile

3.8. Structure-reactivity relationships

The rates of elimination of the amines and the rates of the corresponding reverse reactions are likely to be influenced by steric factors.

The fact that secondary amines are eliminated faster than corresponding primary amines corroborates this hypothesis. Also, atenolol, which is the bulkiest amine tested, eliminates faster than any other compound.

Correlations of reactivity with steric effects have been widely studied by means of the empirical relation:

$$\log\left[\frac{k}{k_0}\right] = a\alpha + C \qquad \textbf{(3.12)}$$

In this equation, k and k_0 represent the rates of a particular reaction involving a molecule with different substituents, a and C are constants and α is a parameter that characterises the molecular structures of the substituents[198].

Taft (E_s) and Charton (ν) parameters are probably the most widely used and they are linearly correlated. They are based in the Van der Waals radii (r_v) of the substituent group: Taft uses the average radii while Charton also considers the symmetry of the group.

Taft's parameter can be calculated by the relation:

$$E_s = -1.839 r_v + 3.484 \quad \textbf{(3.13)}$$

Values are tabulated for several groups, mostly aliphatic and involving carbon chains.

Understandably, there are no tabulated values for most of the model amines and drugs used for preparation of the indenone derivatives studied here and, since the parameters of small groups are not additive, they cannot be used to calculate the parameters of larger groups.

Also, there are not many tabulated values for amines in the literature. Nevertheless, some authors have proposed that the Taft parameter for alkyl groups RCH_2 can be used for correlations of reactions using the corresponding amines RNH_2 (Table 3.8).

Table 3.8: Taft values for model amines

Alkyl group	Es	Amine
n-propyl	-0.36	$NH_2CH_2CH_3$
i-propyl	-0.47	$NH(CH_3)_2$
cC_6H_{11}	-0.79	$cNHC_5H_{10}$
$CH_2CH_2CH_2C_6H_5$	-0.45	$NHCH_2CH_2C_6H_5$

These values do not correlate well with the logarithms of the rates of elimination at basic pH. However, larger rates are observed for bulkier groups. Bad correlation may be due to the fact that elimination from this set of compounds (**C13**, **C12**, **C14** and **C15**) deviated from first order kinetics. Inclusion of other compounds with calculated E_s based on the Van der Waals radii might afford a better correlation but this was not tested.

3.9. Determination of the pKas

The increasing number of papers reporting the determination of pK_as by capillary electrophoresis demonstrates its wide acceptance as an alternative technique to the traditional methods.

The theory of the determination of pK_as by CE is based on the fact that the percentage of ionisation affects the migration velocity of the compound in a particular electrophoretic system. For example, a basic compound should move faster to the cathode as the pH of the running buffer decreases. When fully ionised, the molecule moves faster to the electrode of opposite charge, while in its neutral state it only moves with the EOF. Intermediate effective mobilities (M_e) are a function of dissociation equilibrium and a plot of these against the pH of the running buffer, affords a sigmoidal curve where the inflexion point corresponds to the pK_a. This sigmoidal curve has the form:

$$M_e = \frac{M_a - M_0}{1+10^{(pH-pK_a^{th})}} + M_0 \qquad \textbf{(3.14)}$$

where M_a represents the absolute mobility (cf. Annex 1) and M_0 represents the mobility of the less ionised species, which is evidently zero.

Annex 1 provides a short review on the recent developments and applications of this method of determination of pK_as as well as a fuller development of the theory.

The effective mobilities of the protonated β-aminoketones (BH^+) and some of the corresponding amines were determined by CE.

$$BH^+ \overset{K_a}{\rightleftharpoons} B + H^+$$

The experimental data was then fitted by non-linear regression to equation (**3.14**), affording the pK_as of the different compounds.

Although the accuracy of this method hasn't yet been proved better relative to others, the simplicity of the determination of pK_as by CE is widely acknowledged. This, together with the fact that determination by other methods would be limited due to the instability and low solubility of the compounds, justifies the choice of capillary electrophoresis for the determination of the pK_as of the compounds studied in this work.

Citrate/phosphate/borate buffers with I=0.05 and pH in the range 2-11.5 were used as running buffers. The pH of each running buffer was determined previously and after each set of determinations and the average was taken for the fittings. DMSO or mesytil oxide (MO) were used as markers of the electroosmotic flow. Three consecutive injections were made at each pH.

Mobilities were calculated from the average migration times, the length of the capillary and the applied voltage and were plotted against the pH of the running buffer. Results were fitted to equation **3.14** using GraphPad Prism[196], and the pK_a values were obtained from it. Activity correction for ionic strength ($-log\ \gamma$ =0.083) was applied. Three determinations of the pK_a were made for each compound. Figure 3.35 illustrates the fitting of the sigmoidal plot to the three sets of data of compound **C16**.

2-Aminophenol was tested concomitantly with each batch of tests to ascertain the accuracy of the determinations by evaluation of its two ionisation constants (NH_3^+ and OH) pK_{a1}=4.6±0.2 (n=8) and pK_{a2}=9.8±0.3 (n=6) (lit.[170]: pK_{a1}=4.78 and pK_{a2}=9.97).

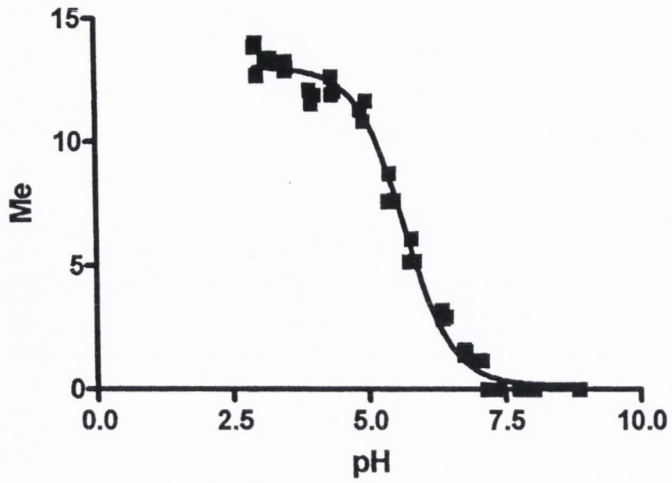


Figure 3.35: Fitting of a sigmoidal curve to the mobility data of C16 for the determination of pK_a

Table 3.9 presents the average and confidence intervals (95%) for the determinations of the pK_as of the β-aminoindanones and respective free amines. Whenever available in the literature, the pK_as of the free amines are also provided.

Table 3.9: Experimental pK_as of the different products determined by CE and pK_as available in the literature

amine	pK_a amine (lit.)	pK_a amine (exp.)	compound	pK_a indanone (exp.)
1-aminoindane	9.19-9.24[199]	9.28±0.16	**C01**	6.48±0.11
			C10	7.51±0.25
2-aminoindane	9.52-9.6[199]	9.32±0.16	**C02**	6.56±0.10
cyclopentylamine	10.7[200]	n.d.	**C03**	7.62±0.16
N,methyl-1-aminoindane*		9.50±0.15	**C04**	5.47±0.10
N,methyl-2-aminoindane*		9.59±0.14	**C05**	5.97±0.16
N,methyl-cyclopentylamine		n.d.	**C06**	6.46±0.25
Dimethylamine	10.73[170]	n.d.	**C12**	6.81±0.06
Ethylamine	10.65[170]	n.d.	**C13**	7.67±0.07
Piperidine	11.12[170]	n.d.	**C14**	7.15±0.08
			C22	7.22±0.08
Phenethylamine	9.83[170]	10.02±0.16	**C15**	6.82±0.05
Atenolol	9.6[170]	9.61±0.63	**C16**	5.75±0.08
Tryptophan methyl ester	7.29[201]	6.89±0.14	**C17**	3.91±0.21
Alanine methyl ester	7.78[201]	n.d.	**C18**	5.04±0.06
Desloratadine		n.d.	**C20**	5.07±0.06
			C23	4.89±0.09
Ephedrine	9.6[170]	9.44±0.77	**C21**	6.16±0.07

* determined from the peaks corresponding to the degradation products of the aminoindanone

The pK_as of the free amines were tested using material of analytical grade except in the case of the products of degradation of compounds **C04** and **C05** where the determinations were made from fully degraded samples of the β-aminoindanones.

For the other compounds determinations were also made based on the peak of the degradation product whenever it was present during the determination of the pK_a of the β-aminoindanone. Consistency between the results obtained for the pK_a from standard material and as degradation product, provided further confirmation of the identity of the degradation products.

In general, a good agreement was observed between the determined pK_as and the pK_as available in the literature. The largest discrepancy was observed for tryptophan methyl ester, but the literature result[201] was reported as an approximation so it might not be accurate.

Reductions of 3-4 pH units were observed for the β-aminoindanones in comparison with the free amines. This is advantageous for prodrug absorption as, at the pH of the intestine, larger amounts of the prodrug are in the neutral form, and consequently available for absorption, in comparison to the corresponding free amine.

These results were in good agreement with the pK_a values obtained by non-linear regression fitting of the pH/rate profiles. Largest discrepancies are observed for compounds where pseudo-first-order kinetics (with or without the equilibrium assumption) did not afford good description of the decay in the concentration of the prodrug (**C21**, **C14** and **C12**).

3.10. Estimation of log P

Log P commonly refers to the octanol/water partition coefficients of molecules and provides a useful estimation of the relative lipophilicities of the compounds.

Generally, for a drug to be suitable for oral administration, its log P should be approximately two. Drugs with lower log P may be badly absorbed unless they make use of one of the available membrane transporters. Drugs with higher log P may be toxic due to accumulation in fat tissue.

The log P of the test compounds were not determined experimentally but they were estimated by a computational method (CS Chemdraw Ultra) in order to evaluate the effect of indanone derivatisation in this parameter. The estimations correspond to the base form.

Table 3.10 presents the results of this estimation of the partition coefficients for the test compounds and for the free amines (in the base form). The literature data available for the amines is also provided.

An increase in the log P of the indanone prodrug was found in comparison with the original amine and it ranged from 1.09 for ethylamine to 1.73 for 2-aminoindane.

Table 3.10: Log P estimations for the test compounds and free amines

amine	log P amine (lit.)	log P amine (estim.)	β- aminoindanone	log P indanone
1-aminoindane		1.00	**C01**	3.01
2-aminoindane		1.00	**C02**	2.73
Cyclopentylamine		0.48	**C03**	1.89
N,methyl-1-aminoindane		1.97	**C04**	3.24
N,methyl-2-aminoindane		1.84	**C05**	3.10
N,methyl-cyclopentylamine		1.00	**C06**	2.26
Dimethylamine		-0.13	**C12**	1.13
Ethylamine	-0.13[c]	0.00	**C13**	1.09
Piperidine	0.60[c]	0.76	**C14**	1.87
			C22	2.43
Phenylethylamine	1.36[c]	1.48	**C15**	2.77
Atenolol	0.008[a], 0.052[b,202]	0.50	**C16**	1.77
Tryptophan methyl ester		0.58	**C17**	2.13
Alanine methyl ester		-0.94	**C18**	0.58
Phenyalanine benzyl ester		2.71	**C19**	4.26
Desloratadine		3.56	**C20**	4.83
			C23	5.39
Ephedrine		1.38	**C21**	2.65

[c] from Chemdraw Ultra database, [a]pH=7, [b]pH=8

3.11. Absorption in rat gut

Based on the lipophilicity data alone, compound **C01** was expected to be better absorbed than the corresponding free amine (1-aminoindane). To evaluate this, the two compounds were tested for absorption in the everted rat gut[203].

A phosphate buffer solution (100 mM, pH=7.4) containing 62 mg/l of compound **C01** was tested and 40-60% of the compound seemed to be absorbed despite some degradation that occurs during the experiment.

The test solution also contained ^{14}C-PEG-4000 that was used to detect alterations in the membrane structure, induced by the test compounds that could affect passive absorption.

Scintillation tests were used to quantify ^{14}C-PEG-4000 and revealed absorption of water in the intestine, since the concentration of the polymer, after perfusion, is higher then before (concentration factor of 1.3). Absorption of **C01** is therefore superior to the 40-60% mentioned before.

The permeability coefficient ρ_{app} (cm/s) can be calculated by the formula:

$$\rho_{app} = \frac{-Q}{2\pi r l} \times \ln \frac{C_l}{C_0} \qquad \textbf{(3.27)}$$

where Q is the flow rate (3.33e-3 ml/s), r is the average rat intestine radius (approx. 0.184 cm), l is the intestine length (approx. 33 cm), C_l is the concentration of the drug at length l and C_0 is the concentration of the drug before administration.

The permeability coefficient for **C01** (corrected for the degradation and concentration effects) is 6.5E-5 cm/s and has a RSD of 8% (4 rats).

Absorption of water was also observed during the administration of 1-aminoindane (30 mg/ml). In this case, a concentration factor of 1.2 was observed. The permeability coefficient for this compound is 3.04E-5 cm/s (average for 3 animals).

2-inden-1-one, the other degradation product of **C01**, as identified later, was not tested individually but was present as a contaminant and was completely absorbed.

3.12. Conclusions

Bibliographic research provided evidence that, although the deamination reaction of β-aminoketones is well known, it has not been used as a prodrug strategy for amines. Scattered references were found where they are referred to act as prodrugs for α,β-unsaturated ketones but these prodrugs consisted mainly of *bis*-Mannich bases (where there are two amino groups in the β-position relative to the ketone). No references for β-aminoketones derivatives of indanone were found.

Single β-aminoketones may have been previously overregarded as prodrug systems due to the relative stability of the compounds towards deamination when compared with the systems containing two amino groups[189].

β-Aminoindanones may eliminate particularly fast when compared with other β-aminoketones due to the high level of conjugation of the enone compound formed upon elimination of the amine.

New model β-aminoketones derived from indanone, prepared in the course of this work, provided confirmation that they degrade to release the free amine at neutral to basic pH while they are generally stable at low pH.

When comparing β-aminoketones derived from primary amines with β-aminoketones derived from the corresponding N-methyl secondary amines, there is a difference in the rates of degradation of more than one order of magnitude. This may be due to steric factors.

Substitution in the α position does not greatly affect the rates of deamination.

Most compounds follow a pseudo-first-order degradation rate in the range of pH tested, however there are some cases where different kinetics is apparent. This was attributed to an equilibrium between the compounds involved. However, this equilibrium is disturbed by the concomitant degradation of 2-inden-1-one.

The equilibrium, should not affect the performance of the compounds as prodrugs, since, due to degradation of 2-inden-1-one, the hydrolysis reaction is eventually driven to completion and also because the volume of distribution of the drug in the body should be enough to avoid a significant reverse reaction.

PK_as were determined by CE. Derivatisation of the amines to produce β-aminoketones reduced the pK_as of the protonated compound by three to four units.

The pH/rate constant profiles for each compound were fitted to a kinetic equation that accounts for spontaneous degradation of the ionised and free base. Non-linear regression afforded estimations of pK_as, which were in fairly good agreement with the pK_as determined by CE.

The partition coefficients of the test compounds and corresponding amines were estimated. They are expected to be higher in the prodrugs than in the original amines, providing for a general better membrane passage. Nevertheless, in some cases they may be too high for oral administration.

As expected the prodrug of 1-aminoindane (**C01**) is better absorbed in the everted rat gut than the free amine. The permeation coefficient for **C01** is about twice that of the corresponding primary amine. Some diuretic effect is observed.

In general, at physiological pH, the compounds degraded, with half-lives that ranged from 3 minutes to seven hours and are consistent with their application as prodrug systems. On the other hand, the compounds were stable in acidic medium. The promoiety should impart a stronger lipophilic character to the compound and the new molecules have lower pK_as

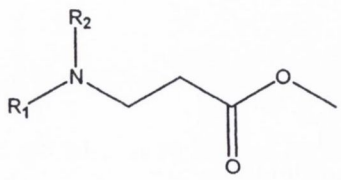
than the original amines. Both this characteristics should contribute to a better passage through biological membranes. Experimental evidence of a better absorption of the prodrugs in the everted rat gut arose from tests with 1-aminoindane.

CHAPTER 4. OTHER β-AMINOKETONES AS PRODRUGS

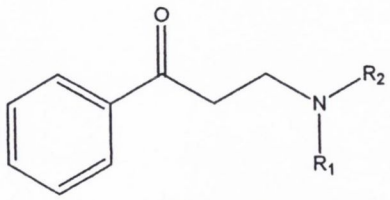
4.1. Introduction

Chapter 4 describes the preparation and testing of β-aminoketones derived from different ketones. The main goal was to compare them with the previously studied indanone system, in terms of ease of synthesis, rate of amine release and stability. We were also interested in determining if the behaviour of the indanone system was unique and if so, what specific structural features promoted amine release.

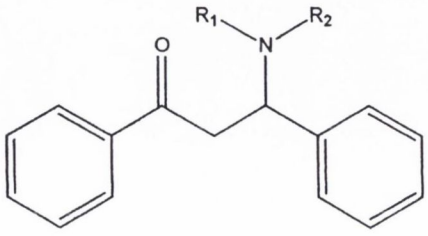
Four new systems were studied: methyl propionate, methyl acetophenone, benzyl acetone and benzyl acetophenone.



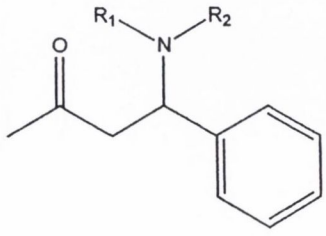
methyl propionate



methyl acetophenone.



benzyl acetophenone



benzyl acetone

The synthesis strategy was dictated by the target compound. This chapter will provide a short review of the reactions usually utilised in the preparation of β-aminoketones (C-Mannich bases).

Section 4.3 is dedicated to describing the attempts made for the synthesis of the new compounds.

In general, the 3-amino propionates were prepared by substitution of the bromine from 3-bromo propionic acid methyl ester, which was commercially available. Amino derivatives of methyl acetophenones were prepared by the Mannich reaction. Since, in amino benzyl acetones, the amine is in the benzylic position, *Wohl-Ziegler* bromination was initially attempted in order to prepare bromine derivatives that would afterwards be substituted by the intended amine. This route proved unsuccessful and instead the synthesis was carried out by Michael addition of the amines to the respective unsaturated ketone (after some frustrated attempts to use the Mannich reaction). The Mannich reaction was initially employed for preparation of amino benzyl acetophenone but ultimately, direct Michael addition to chalcone (the corresponding unsaturated ketone) was preferred.

The compounds were then tested for retro-Michael reaction and this work is described in section 4.4. The pH/rate profiles of degradation of compounds obtained with suitable purity, were determined (37°C, I=0.154) and fitted to appropriate kinetic models by non-linear regression in the manner described in the previous chapter.

The pK_as obtained using this procedure were compared with the pK_as determined by CE from the effective mobilities of the compounds at different pH.

4.2. On the preparation of β-aminoketones

At the beginning of the XXth century, β-aminoketones were prepared by condensation of imines and ketones in ethanol[174].

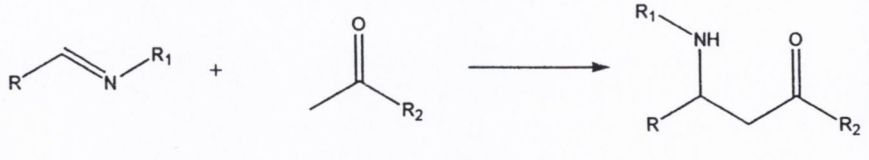


Figure 4.1: Preparation of β-aminoketones from imines and ketones

The reaction was initially thought to occur only with methyl ketones but it was later observed that other alkyl ketones would also react in a similar manner[204]. Today this type of reaction is performed with iminium salts and using catalysis by a Lewis acid to promote enolisation of the ketone with silyl enolates[205]. By using selected catalysts it is possible to

promote enantioselective reactions[206]. Kobayashi *et al.*[207] provide a good review on this type of reaction.

These reactions are enhancements of the well-known C-Mannich reaction that was first described in 1935[208]. The product of a Mannich reaction is usually called a Mannich base and, depending on the type of bond formed, can be a C, N, O, or S-Mannich base[182]. A C-Mannich reaction, involving a ketone as the substrate, is illustrated in Figure 4.2.

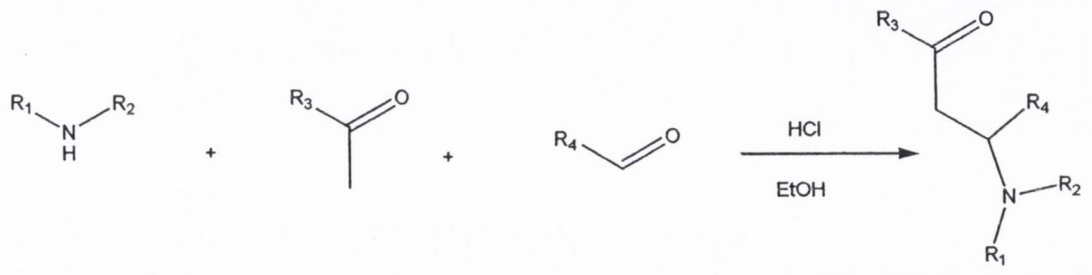


Figure 4.2: C-Mannich reaction

The acid-catalysed reaction may be carried out in a "one pot", three component reaction, usually in ethanol[209,210] or DMSO[211] but there are also good results reported for Brønsted acid-surfactant-combined[212] and Lewis acid[213] catalysis in water. However, amines used in both cases were aromatic. Base catalysis can also be used[149].

In the most common C-Mannich reaction, acetophenone and formaldehyde are refluxed in ethanol with HCl to produce 3-amino-1-phenyl-propan-1-ones (aminomethyl acetophenones), but if benzaldehyde is used instead, it is possible to produce 3-amino-1,3-diphenyl-propan-1-ones (aminobenzyl acetophenones). This reaction leads to several side products and has some substrate limitations[149,210,212]. Some alkyl carbonyl compounds may need to be stabilised in the enolic form, either by silyl, metal or boron enolates[182].

There are few limitations in the choice of the amine reactant, except for the unreactive tertiary amine derivatives[182]. Although most authors use the salt form of the amine, the proposed mechanisms involve the reaction of the amine in the free form[149].

The usual practice still involves mixing the reactants of Mannich synthesis with or without following a particular order of addition but the use of preformed aminomethylating reagents[214] like methyleneammonium salts is becoming more frequent[182]. Benzotriazone adducts obtained from condensation with the carbonyl compound and the amine, have also been used[215].

Two mechanistic routes are possible for the classic Mannich reaction as illustrated in Figure 4.3. Generally path **a** is considered the preferred one but in some cases, depending on the reactivity of the substrate, path **b** may dominate.

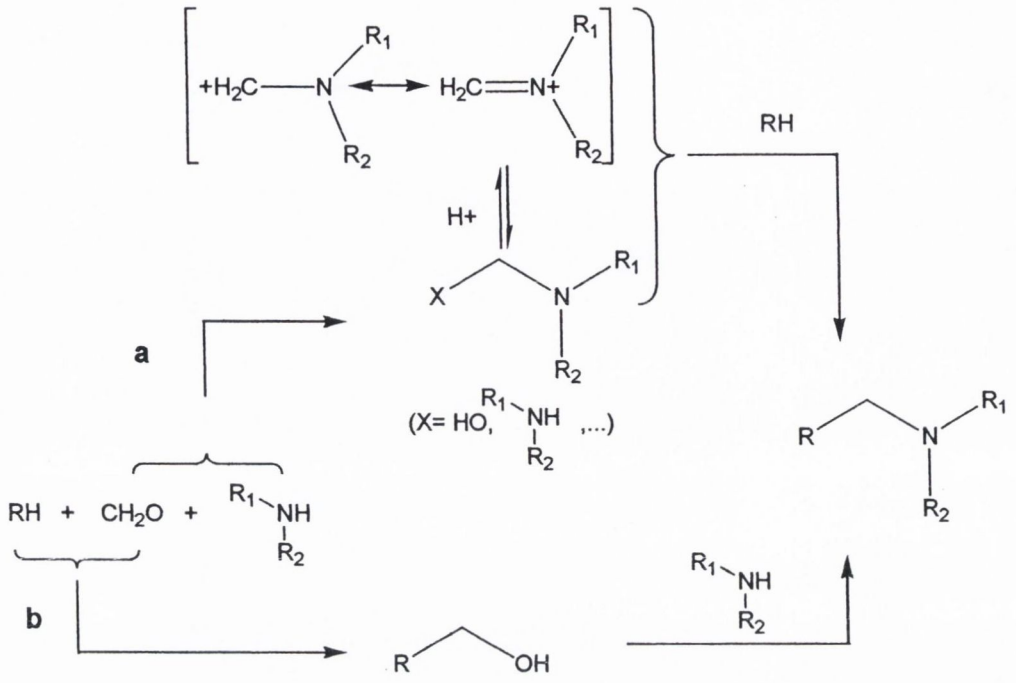


Figure 4.3: Mechanistic routes of the Mannich reaction[182]

A base catalysed mechanism has also been proposed[149].

Many authors have described difficulties with Mannich reactions, with several additional side products being formed. If two active hydrogens are available in the substrate, for example, the addition of two alkylamines is possible, as in the case of the reaction between acetophenone, formaldehyde and piperidine[215].

Another common approach for the synthesis of β-aminoketones, esters and acids, is the direct Michael addition of the amine to the α,β-unsaturated carbonyl compound (Figure 4.4).

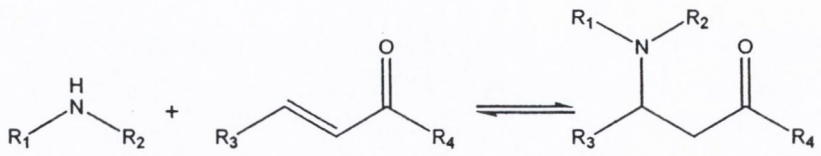


Figure 4.4: Michael addition of amines to unsaturated carbonyl compounds

This reaction has been cited many times in the literature. Several different methods have been detailed, such as solid state reactions[216], reaction in organic solvent[216,217,218,219,220,221] with or without base catalysis, surfactant-assisted aqueous reactions[178] and reactions under microwave radiation[222]. The same type of reaction, which is reversible, is observed between an α,β-unsaturated ketone and methoxylamine in alcohol. If the amine is primary, the addition of two ketones is possible[223].

Due to the above-mentioned equilibrium, it is frequently difficult to produce compounds in high yields and purity. Surfactant-assisted aqueous reactions provide an interesting alternative for the production of compounds with relatively high purity[178]. Reactions under microwave radiation[222] involve the use of a large excess of the amine that is subsequently removed by evaporation and are, for this reason, restricted to volatile amines.

Grignard reagents can also be used to promote conjugate addition to α,β-unsaturated carbonyl compounds and this approach has been used in the synthesis of peptide mimetics. If the addition of an amine is desired, an organometallic amide is firstly prepared at low temperature from the corresponding amine and a Grignard reagent. The resultants adduct is then added to the unsaturated carbonyl compound, usually at room temperature. Diethylamine has been added to chalcone in this manner, using the corresponding titanium amide[224].

A more recent approach in the production of β-aminoketones involves the reduction of enaminones. Moreover, a reduction catalyst also facilitates the direct Michael addition of amines to enones and, since enones are common secondary products in the Mannich reaction, it is also possible to increase the yields of aminoketones in these reactions by addition of a reduction catalyst like alumina[225].

4.3. Preparation, and identification of the test compounds

4.3.1. Propionates

3-Bromopropionic acid methyl ester was used to prepare the derivative of desloratadine (**C24**) and 2-aminoindane (**C25**) by nucleophilic substitution, in the same manner as the previously described reaction of 3-bromoindanone with different amines (Figure 4.5).

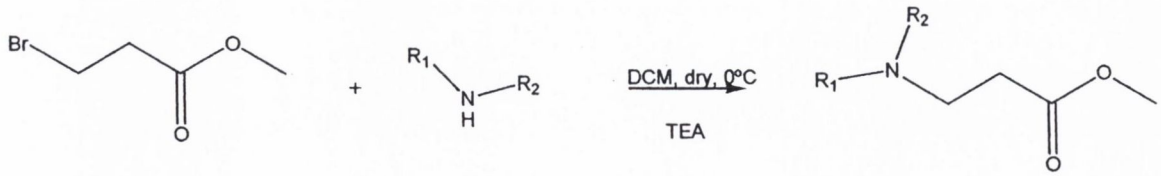
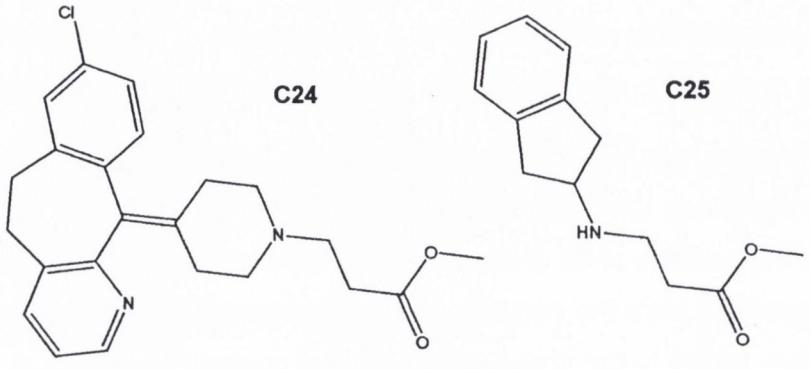


Figure 4.5: Preparation of amine derivatives of methyl propionate

In this case, only UV absorbing amines could be used so that the compounds could be detected in the CE and HPLC systems.

The target compounds were isolated and their identity was confirmed by NMR, IR and MS (Chapter 7).



C24 C25

4.3.2. Methyl acetophenones

Methyl acetophenones were prepared by the Mannich reaction[182,209] in ethanol using acetophenone as the substrate and formaldehyde as the alkyl extension (Figure 4.6).

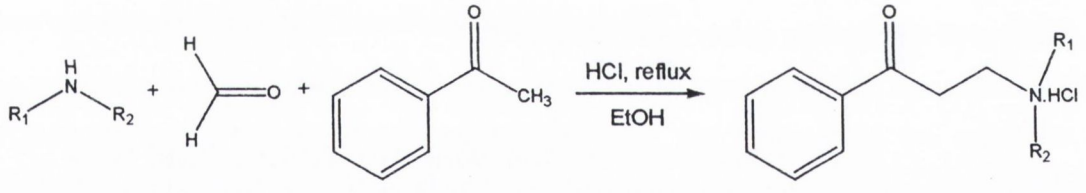
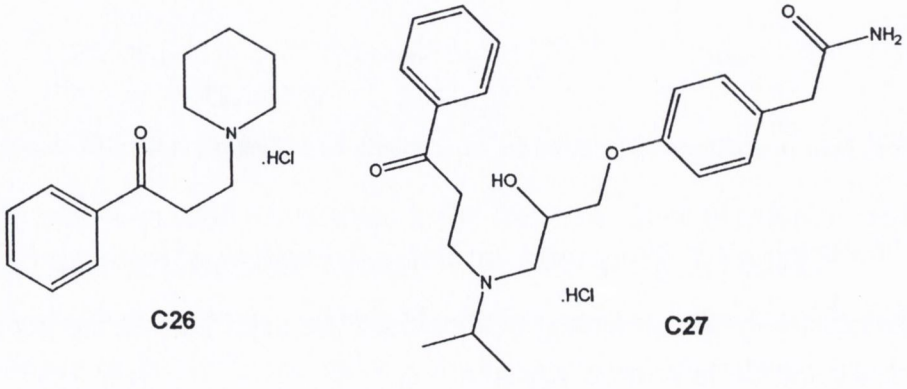


Figure 4.6: Mannich reaction applied to the preparation of aminomethyl acetophenones

The target products were the derivatives of piperidine (**C26**) and atenolol (**C27**). Neither product was obtained pure or could be unambiguously identified. This reflects difficulties previously encountered by other authors with the Mannich reaction[215].



C26 C27

The amino derivatives of methyl acetophenones were meant to be examined in order to evaluate if the position of the double bond in the conjugated aromatic ring/double

bond/carbonyl system would influence the rates of degradation. Due to the difficulties with their synthesis and, since these compounds may not be suitable for clinical development due to the known mutagenicity of the vinyl ketone chemical class[188], tests with these compounds were discontinued.

4.3.3. Benzyl acetones

The synthesis of the aminobenzyl acetones was initially attempted by means of a two step reaction, starting with the bromination of 4-(4-hydroxyphenyl)-2-butanone, the only benzyl acetone available commercially at the time. The second step would involve the nucleophilic substitution of the bromine by the intended amine.

Bromination was attempted using bromosuccinimide and dibenzoyl peroxide in carbon tetrachloride (as in the case of the derivatives of indanone) but this procedure was unsuccessful as the reaction mixture turned black and no product could be isolated from it. The use of different radical initiators such as 1,1-azobiscyclohexane-carbonitrile or light also failed to facilitate the synthesis of the desired product.

The reaction products were not identified but these difficulties were thought to be due to oxidation of the phenol group (Figure 4.7).

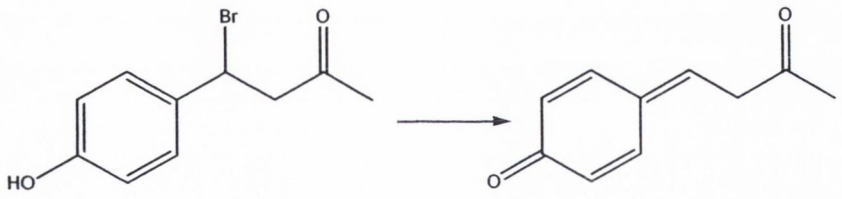


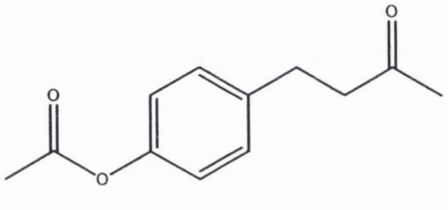
Figure 4.7: Oxidation of the brominated product of 4-(4-hydroxyphenyl)-2-butanone

For this reason, protection of the labile phenol group was performed before attempting further bromination. Acetylation was performed using acetic anhydride in dry DCM and in the presence of TEA. The reaction mixture was washed with solutions of 0.01 M potassium hydrogen carbonate and 0.1 M HCl before evaporation. The product (**B07**) was identified by NMR and IR.

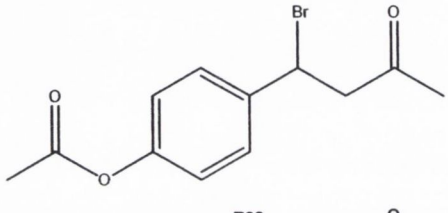
Bromination of this product was attempted by the usual method but the final product (**B08**) was not isolated despite the fact that it was produced at a certain stage during the reaction as confirmed by NMR analysis of the reaction mixture. During evaporation of the solvent, the mixture turned black as was observed during synthesis of the hydroxyl derivative.

Protection was also attempted using trimethylsilylation (**B09)** but the bromination product (**B13,** chapter 7) could not be isolated although there was some evidence (GCMS) that it was produced.

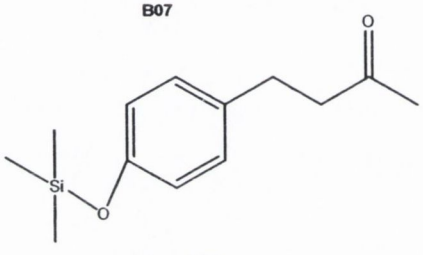
Protection by methoxylation afforded compound B10. Partial bromination was achieved (B14, Chapter 7), as confirmed by GCMS of the reaction mixture, but it was not possible to isolate the compound.



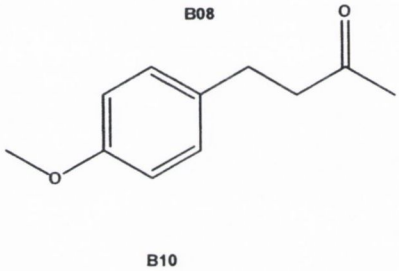
B07



B08

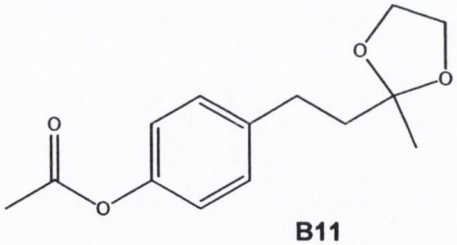


B09

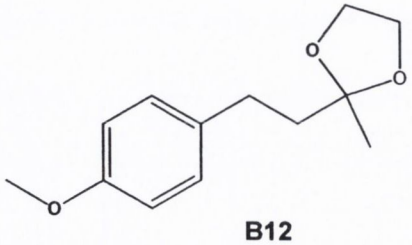


B10

Simultaneous protection of the ketone and the hydroxyl groups was also performed in an attempt to reduce the reactivity towards elimination of the bromine. For this purpose, ethyleneglycol was used in the presence of p-toluenesulphonic acid in toluene under dry conditions (Dean-Stark) followed by protection of the hydroxyl group by methoxylation or acetylation (**B11** and **B12**).



B11



B12

Bromination of these products did not appear to be successful since succinimide, which should form during this reaction and be visible in the form of a pale solid floating in the reaction mixture, was not noted.

Mannich reactions were also attempted in the synthesis of some amino benzyl acetones, using acetone as the substrate, benzaldehyde as the linkage group and piperidine or 1-aminoindane as the amines (Figure 4.8).

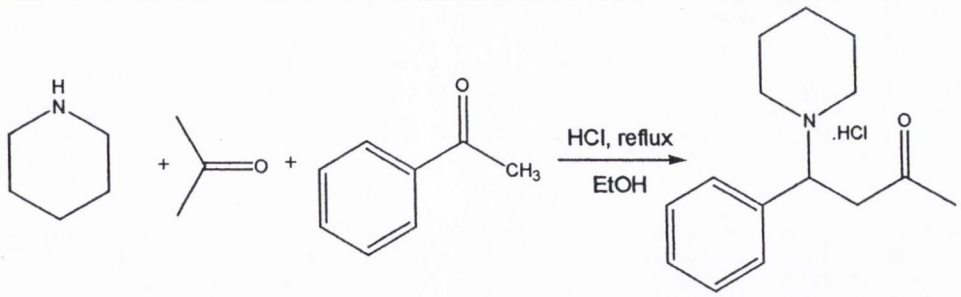


Figure 4.8: Mannich reaction for the preparation of benzyl acetones

The reaction proved unsuccessful as the salt of the original amines were recovered in the hydrochloride form.

Following these unsuccessful attempts, a new synthesis strategy was tested which involved direct Michael addition of the amine to the unsaturated ketone (benzalacetone) (Figure 4.9).

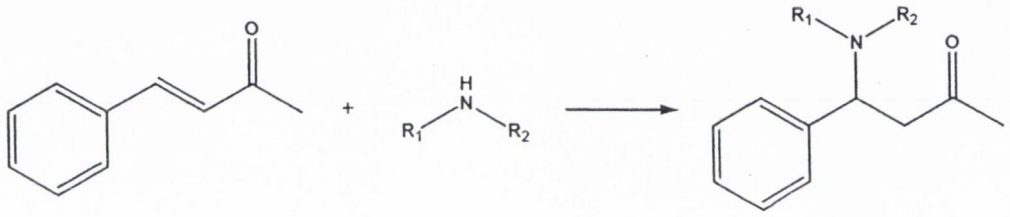
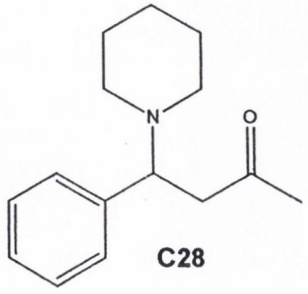


Figure 4.9: Preparation of aminobenzyl acetones from benzalacetone

Several different conditions were investigated in order to find the best performance as described for each product. Different solvents were tested but the reaction was more successful when liquid amines were added to benzalacetone in the absence of solvent. In the case of piperidine, compound **C28** was obtained as a clear oil as the sole product of the reaction and did not require any further 'clean-up'.



C28

Liquid primary amines reacted in solventless conditions but yielded mixtures of products which proved impossible to isolate as they degraded during clean up by flash chromatography. Usually three peaks were observed by CE. They could be due to the compound resultant from single addition and to the pair of enantiomers and the meso compound resultant from the double addition of benzyl acetone (Figure 4.10).

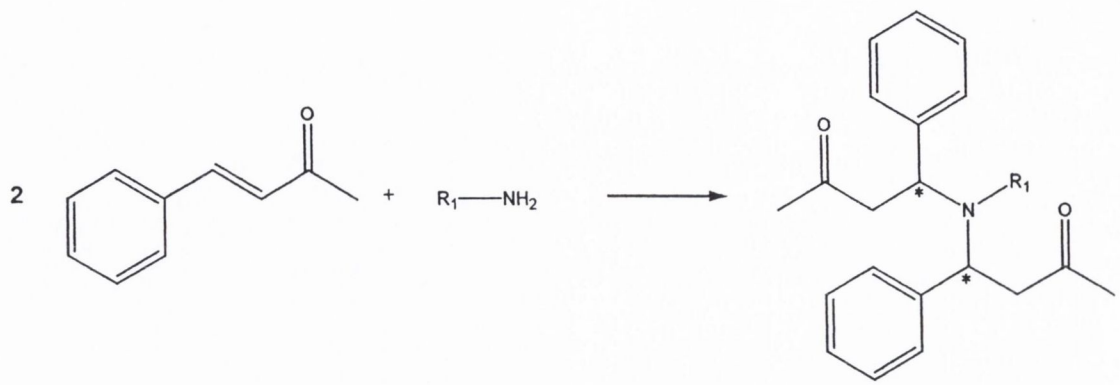
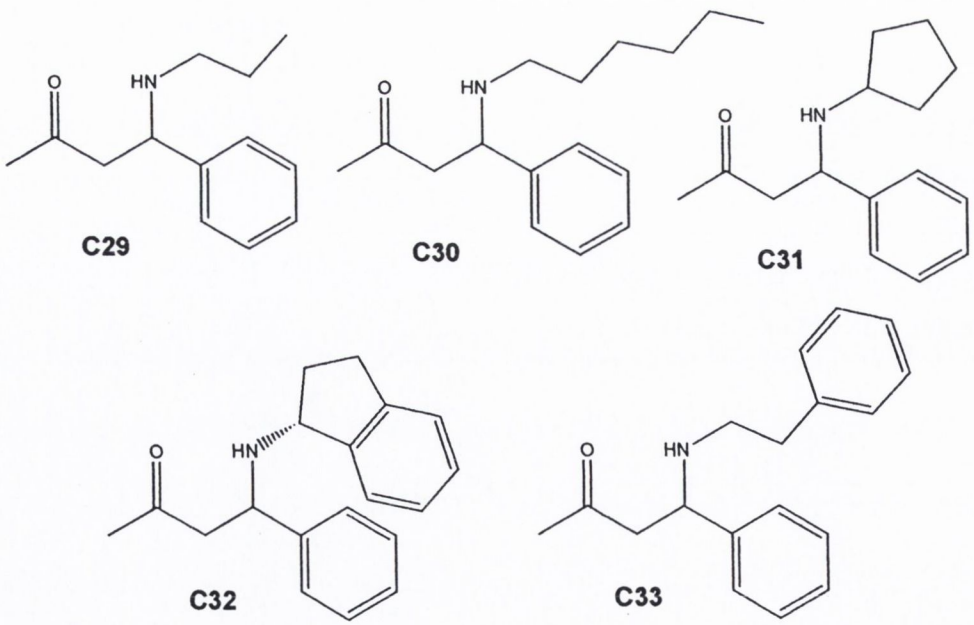


Figure 4.10: Addition of two molecules of ketone to primary amines

In the case of the derivatives of phenylethylamine and 1-aminoindane, significant amounts of unreacted amines were detected by CE.

This situation might have arisen when attempting to obtain the derivatives of N-propylamine (**C29**), N-hexylamine (**C30**), cyclopentylamine (**C31**), *R*-1-aminoindane (**C32**) and phenylethylamine (**C33**).



4.3.4. Benzyl acetophenones

The Mannich reaction was also attempted for the synthesis of aminobenzyl acetophenones. In this case, benzaldehyde was used instead of formaldehyde (Figure 4.11).

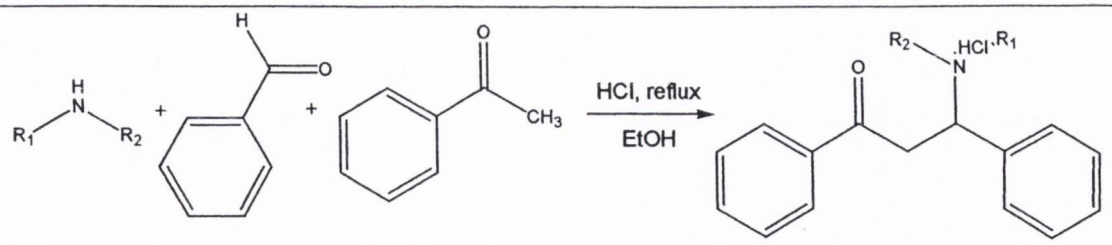
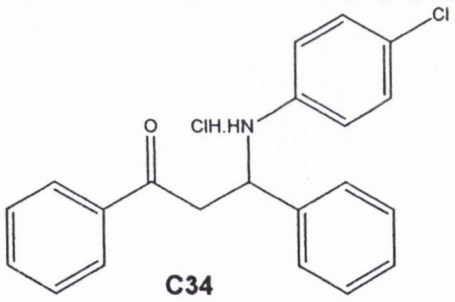


Figure 4.11: Synthesis of aminobenzyl acetophenones by the Mannich reaction

Typically, equimolar concentrations of the ketone, aldehyde and the amine were refluxed in ethanol for over 2 hours under acidic conditions (1eq. of HCl) and then the mixture was allowed to cool slowly. The precipitated solid was recovered by filtration.

This reaction is only mentioned in the literature as applied to derivatives of benzylic amines and despite being tested with different amines during the present study, only compound **C34** could actually be obtained through this reaction. When piperidine or 2-aminoindane were used, the hydrochloride salts of the amines were recovered unreacted.

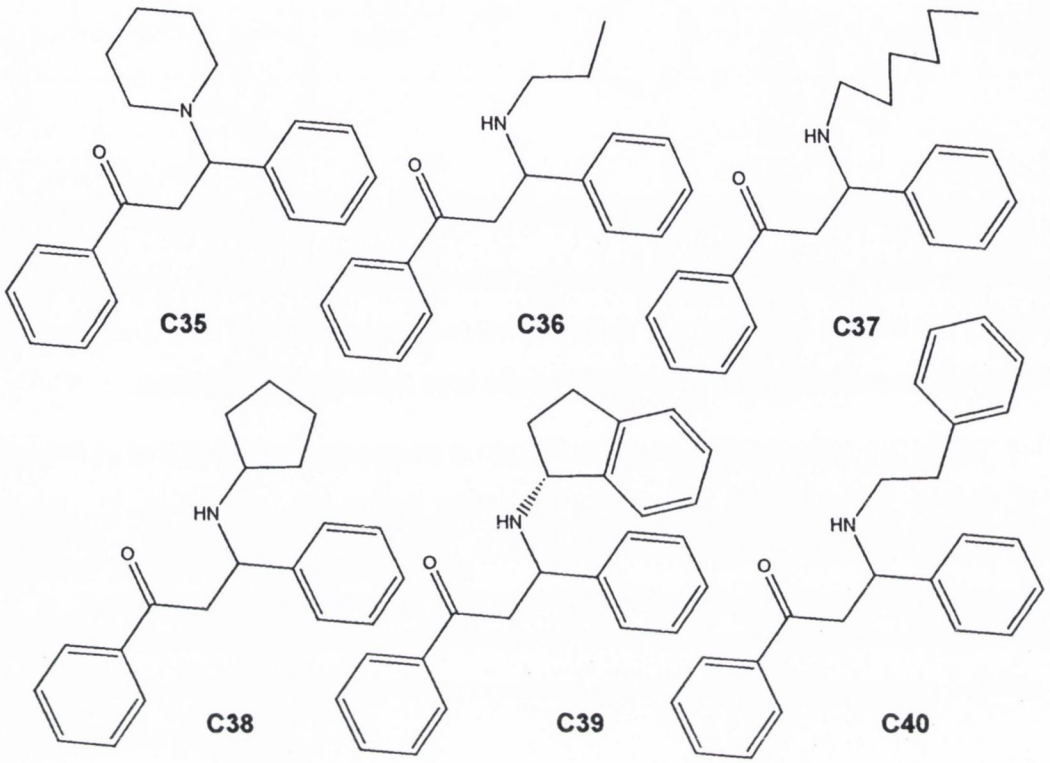


C34

Direct Michael addition to benzalacetophenone (chalcone) was also attempted in different solvents (hexane, pyridine, cyclohexane/ether/potassium carbonate) in reflux. In most cases, no new spots were observed by TLC but some product was formed in the case of compound **C35**.

This reaction was most successful when equimolar quantities of a liquid amine and chalcone were added and homogenised at room temperature in solventless conditions. Typically, the original liquid mixture would solidify in less than 10 min to afford the desired compound quantitatively. In this case, and in contrast to what happens with benzalacetone, only one product was obtained even in the case of primary amines. This may be a result of steric factors.

Derivatives of piperidine (**C35**), N-propylamine (**C36**), N-hexylamine (**C37**), cyclopentylamine (**C38**), *R*-1-aminoindane (**C39**) and phenylethylamine (**C40**), were obtained by this procedure.

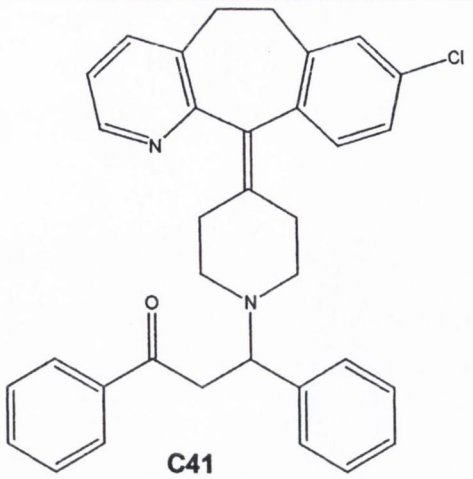


The compounds were generally obtained as white solids or oils. In most cases the oils solidified when returned to room temperature after retention for a period in the ice compartment of the refrigerator.

Ultimately all compounds solidified except **C39**, which was obtained as a yellowish oil. This may be explained by the fact that the reaction between the amine (which already has a chiral centre) and chalcone affords a mixture of two diastereomers due to a new chiral centre arising from the covalent bond between the amine and chalcone.

Desloratadine did not react through this process but it was possible to obtain a new product (detected by TLC) by reaction in cyclohexane/ether (1:2) and potassium carbonate. Attempts to clean this compound by flash chromatography were unsuccessful, as the compound degrades rapidly during the 'clean-up' procedure.

A reaction was also attempted in aqueous solution in the presence of a surfactant (hexadecyltrimethylammonnium bromide)[178]. Initially there was no evidence of reaction, but after addition of a small quantity of acetonitrile and ethanol to help solubilise the chalcone, a new product started to precipitate, which, after drying, proved to contain the target derivative of desloratadine (**C41**) in the salt form, which was, however, contaminated with chalcone.



C41

4.4. Elimination of amines from the test compounds

The compounds were tested at 37°C and I=0.154 in order to evaluate the kinetics of degradation and the effect of the carbonyl structure on reactivity.

Where applicable, the pH/rate coefficient profiles were fitted by non-linear regression to equation **3.9** and the pK_as of the compounds were derived from it.

4.4.1. Propionates

3-Amino propionic acid methyl esters were tested in order to evaluate the effect of the absence of the aromatic ring, as well as the feasibility of using an ester instead of a ketone as the carbonyl component.

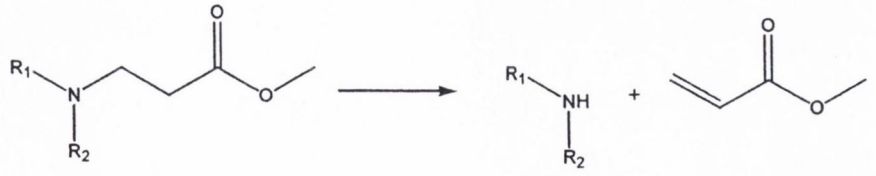


Figure 4.12: Deamination reaction of 3-amino propionates

The reaction under investigation is depicted in Figure 4.12. However, both products (**C24** and **C25**) degraded in buffered solutions but not to the original amines as only a small peak appeared at the migration time of desloratadine and none at the retention time of 2-aminoindane. The major degradation products were detected by CE, in both cases, at higher migration times than the aminopropionates. This could be attributable to the formation of the corresponding acids (Figure 4.13).

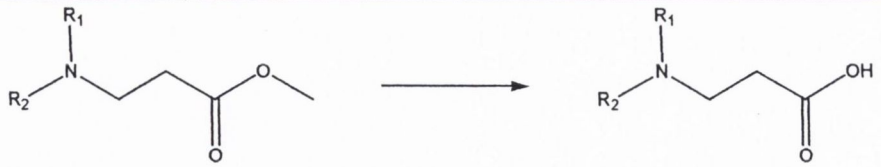


Figure 4.13: Hydrolysis of 3-amino propionates

These products could have a migration time higher than the esters, depending on the pK_a of the acid. If the acid group is ionised as well as the amino group at the pH of the running buffer (which is less than four), the species is neutral and only moves by the action of the EOF; at the same pH, the amine function of the ester is protonated and for that reason moves faster towards the cathode.

Deamination of the acid is not likely to occur. This was confirmed by the fact that free amines were not detected.

4.4.2. Benzyl acetones

Benzyl acetone derivatives are quite similar to indanone derivatives in terms of the possibility of conjugation following elimination of the amine. However, this system is not as structurally restrained. This may have some influence on the rate of the reverse reaction as benzalacetone has two protons available for substitution while indenone has only one. Moreover the two protons may not be equal with respect to stereochemical environment.

The general deamination reaction is depicted in Figure 4.14.

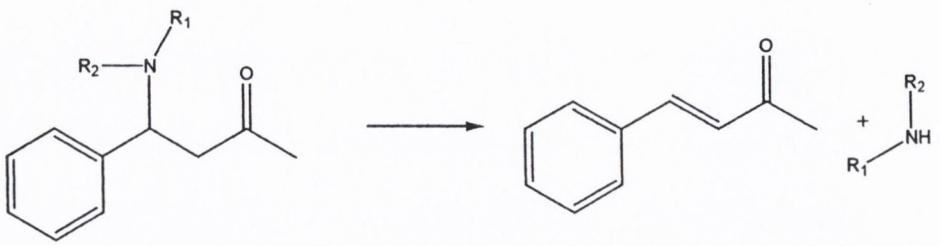


Figure 4.14: Deamination reaction of amino benzyl acetones

4.4.2.1. Piperidine (C28)

Solutions of **C28** (3 mM) were tested at four different pH values. The compound was found to be stable at low pH and degrades with a half-life of less than 3 min at neutral to basic pH.

The pseudo-first-order curves for the disappearance of the compound in various solutions are depicted in Figure 4.15.

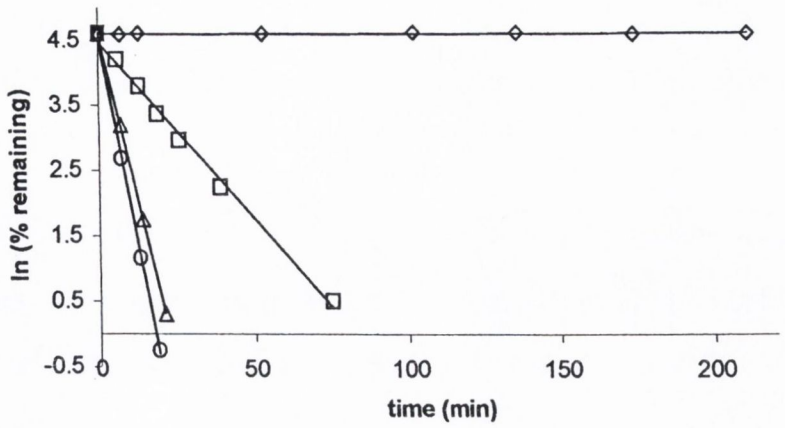


Figure 4.15: Pseudo-first-order curves for the disappearance of C28

(◊ pH=3.0 □ pH=6.2 □ pH=7.8 ○ pH=11.6)

This data was fitted by non-linear regression to equation **3.9**, which afforded the sigmoidal line in Figure 4.16. The point at pH=11.6 was excluded due to the possible different mechanism of reaction.

The degradation rate at basic pH afforded by this procedure is 0.21 min^{-1}, which corresponds to a half-life of 3.3 min. However, the small number of experimental points may affect the accuracy of this determination.

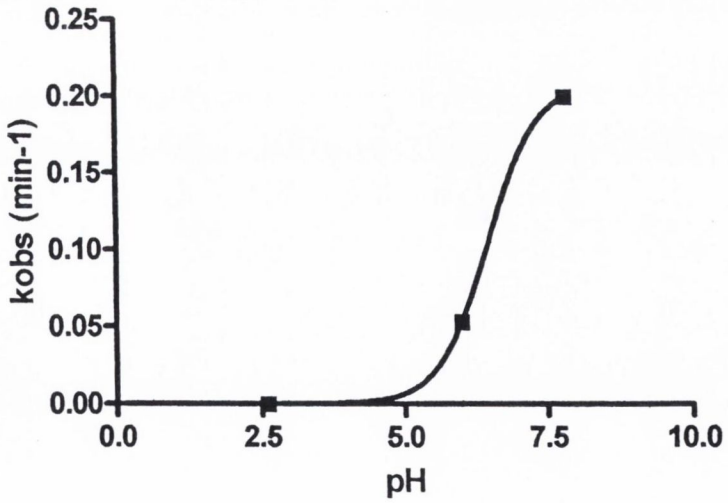


Figure 4.16: Fitting of equation 3.9 to the pH/rate profile of C28

4.4.2.2. Primary amines

The electrophoregrams of the crude mixtures of primary amines with benzalacetone generally exhibited three new peaks, which could be due to the formation of compounds resultant from one or two additions of the ketone to the amine. This would afford compounds with multiple chiral centres.

All the compounds generally degraded with pseudo-first-order rates and with the concomitant increase in the areas of the original amines (whenever detectable). The rates of degradation were lower at acidic pH.

The rates of degradation of the main two peaks of each compound at pH=7.4 are in Table 4.1 evidencing fast degradation.

Table 4.1: Degradation rates and half-lives of the two main peaks of amino benzyl acetones derived from primary amines (pH=7.4)

	k_{obs} (min^{-1})	$t_{1/2}$ (min)
C29	0.028, 0.029	24, 25
C30	0.037, 0.024	19, 28
C31	0.34, 0.083	2, 8
C32	0.060, 0.015	11, 46
C33	0.069, 0.041	10, 17

4.4.3. Benzyl acetophenones

Degradation rates in benzyl acetophenone derivatives are quite high and this may be due to the high level of conjugation introduced by elimination of the amines from the test compounds as illustrated by the general deamination reaction in Figure 4.17.

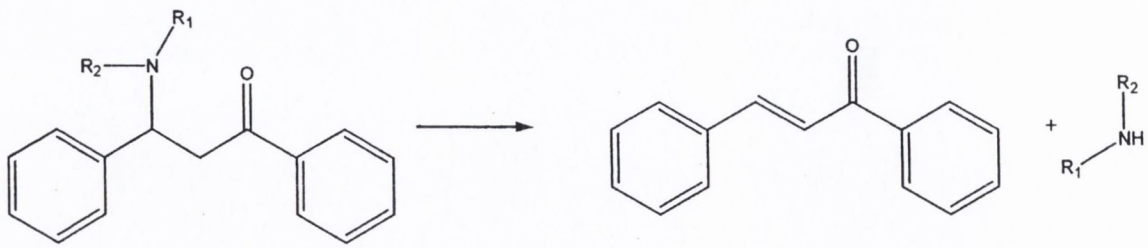


Figure 4.17: Deamination reaction of amino benzyl acetophenones

The reverse reaction may also be more favoured in the case of indanone derivatives due to less stereochemical hindrance (indenone is always in *cis* conformation while chalcone may be *cis* or *trans*).

Additionally, elimination may be particularly favoured in chalcone derivatives, through a cyclic six-membered transition state, where the ketone is in the enolic form (Figure 4.18).

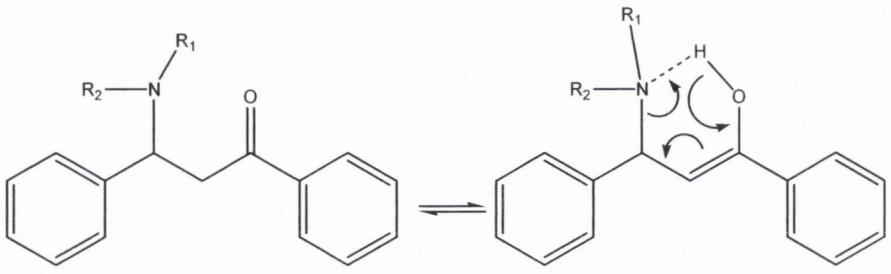


Figure 4.18: Cyclic six-membered transition state for the elimination from benzyl acetophenone systems

The position suggested for the enol hydrogen is favoured by hydrogen bonding and the lone-pair electrons on nitrogen are potentialiy leaning towards oxygen, since the amine substituents should be pointing outwards due to steric effects. A similar mechanism has been suggested by other authors for the retro-Michael reaction of methyl acetophenones[183].

4.4.3.1. Piperidine (C35)

The benzyl acetophenone derivative of piperidine was found to be more unstable at low pH than the benzyl acetone analogue. At pH 2.6, its half-life is 10.5 hours while at neutral to basic pH, the half-life of this compound is between five and eight minutes. At all pHs the degradation followed pseudo-first-order kinetics (Figure 4.19).

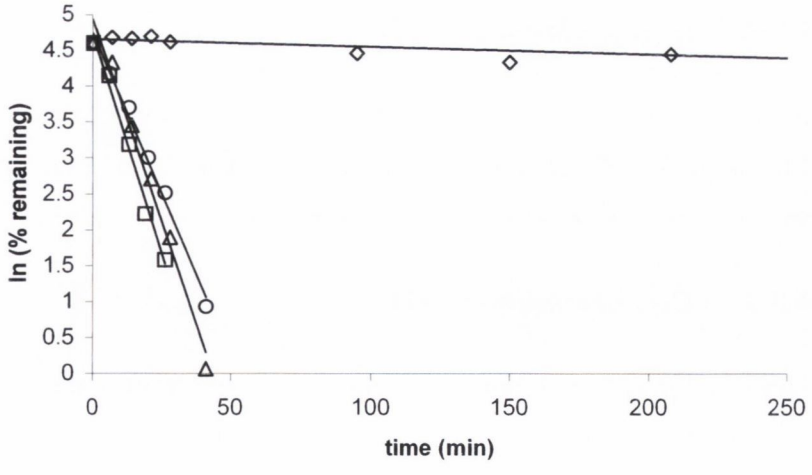


Figure 4.19: Pseudo-first-order curves for the disappearance of C35

(◊ pH=3.0, □ pH=6.2, Δ pH=7.8, ○ pH=11.6,)

In comparison with the other carbonyl derivatives, benzyl acetones and benzyl acetophenones seem to be much less stable as illustrated in Figure 4.20.

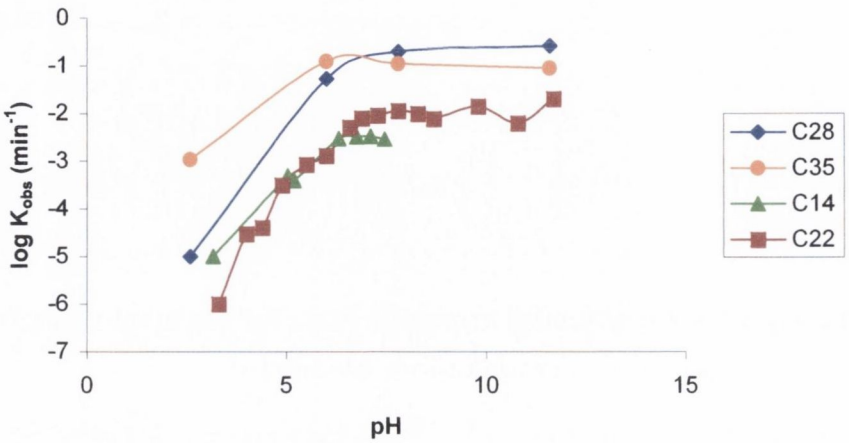


Figure 4.20: pH/rate profiles of the derivatives of piperidine

Table 4.2 provides the rates of degradation at basic pHs as afforded by fitting equation **3.9** to the pH/rate profiles of the different compounds.

Table 4.2: Rates of degradation and half-lives of derivatives of piperidine

	k_{obs} (min^{-1})	$t_{1/2}$ (min)
C14	0.0033	210
C22	0.012	58
C28	0.21	3.3
C35	0.12	5.8

4.4.3.2. N-propylamine (C36)

Compound **C36** was tested at pH=2.3 and pH=7.4. It was found to be stable in acidic conditions and to degrade with pseudo first-order kinetics (k=0.052 min^{-1}, $t_{1/2}$=13 min.) at physiological pH.

4.4.3.3. Desloratadine (C41)

Aqueous buffered solutions of compound **C41** were prepared from a stock solution in DMF or DMF/ACN mixtures since it was not soluble in ACN alone.

Even in this case the peak detected by CE was very large and unsymmetrical and was difficult to quantify, probably as a result of low solubility. Nevertheless, upon analysis of the solutions of this compound in the pH range 2.8-11.2, the formation of desloratadine was observed at all pHs. Production of desloratadine seemed to be higher at low pH but this

probably does not reflect the differences in the degradation rates at different pHs since low solubility may limit degradation at higher pH.

4.4.3.4. N-hexylamine (C37)

Compound **C37** was tested in the pH range 0.5-11 and was found to be stable under acidic conditions. At pHs higher than three, the compound degrades with pseudo-first-order degradation rates with the following pH/rate profile (Figure 4.21).

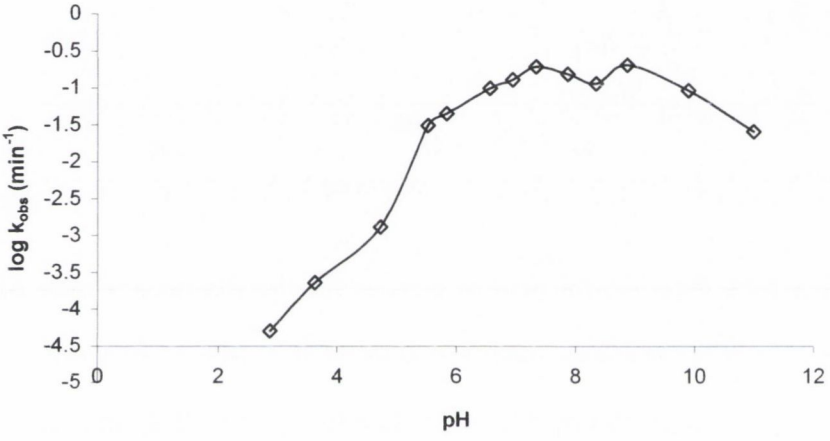


Figure 4.21: pH/rate constant profile of compound C37

The data used for this profile at pHs lower than eight was fitted to equation **3.9** and afforded the sigmoidal curve displayed in Figure 4.22.

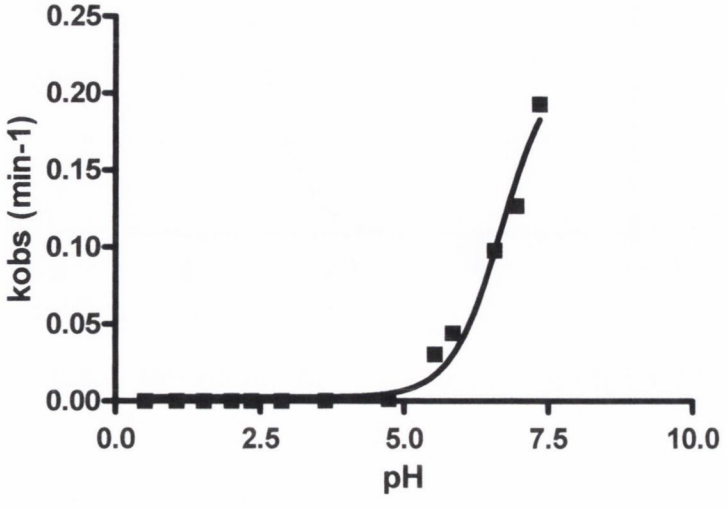


Figure 4.22: Fitting of equation 3.9 to the pH/rate profile of C37

4.4.3.5. Cyclopentylamine (C38)

The cyclopentylamine derivative is stable in acidic medium and degrades following pseudo-first-order kinetics at higher pH (Figure 4.23).

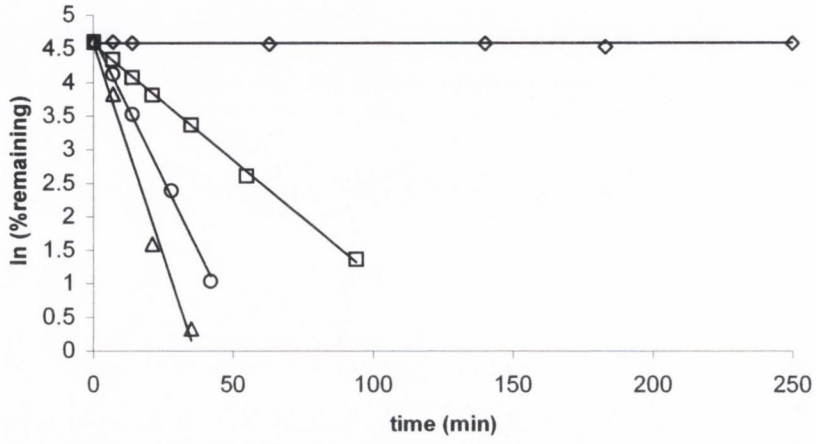


Figure 4.23: Pseudo-first-order curves for the disappearance of C38

(◊ pH=3.0, □ pH=6.2, Δ pH=7.8, ○ pH=11.6)

A sigmoidal curve was obtained by fitting the data to equation **3.9**, after excluding the point at the highest pH (Figure 4.24).

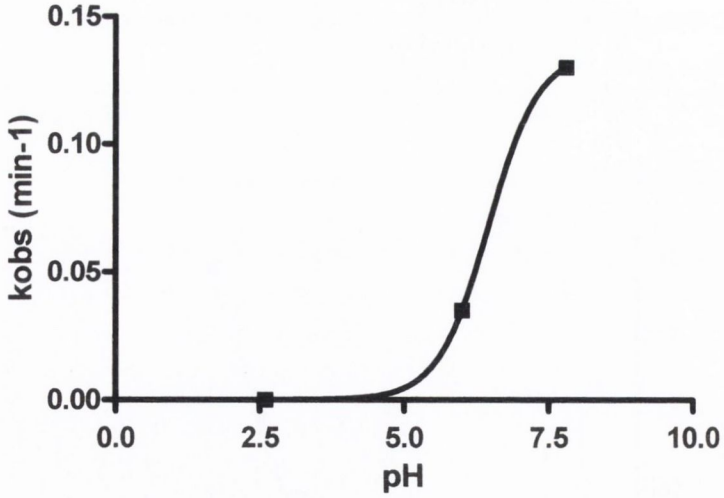


Figure 4.24: Fitting of equation 3.9 to the pH/rate profile of C38

In comparison to the other derivatives of cyclopentylamine, compound **C38** degrades at much faster rate than the indanone derivative (**C03**) and at comparable rates to compound **C06**, which is the tertiary analogue of the indanone derivative (Figure 4.25).

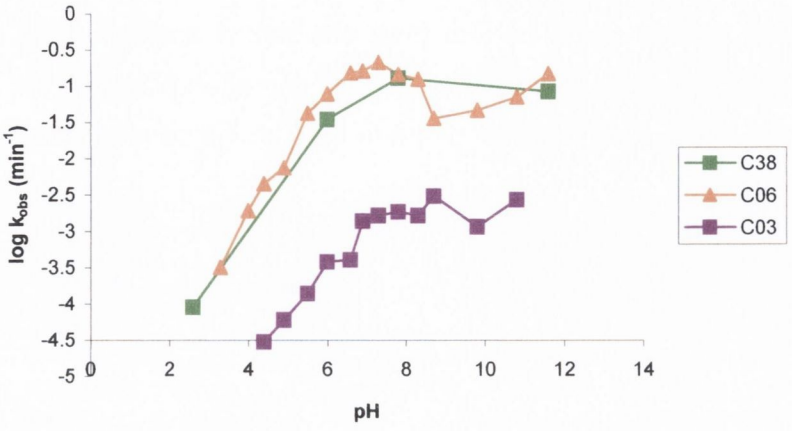


Figure 4.25: pH/rate profiles of the derivatives of cyclopentylamine

The degradation rates afforded by non-linear regression and the corresponding half-lives are presented in Table 4.3.

Table 4.3: Rates of degradation and half-lives of the derivatives of cyclopentylamine

	k_{obs} (min^{-1})	$t_{1/2}$ (min)
C03	0.0024	288
C06	0.22	3.2
C38	0.14	5.0

4.4.3.6. R-1-aminoindane (C39)

Compound **C39** degraded with the concomitant release of 1-aminoindane. At pH=7.8 the half-life was 13.6 minutes while at pH=3 it was over five days.

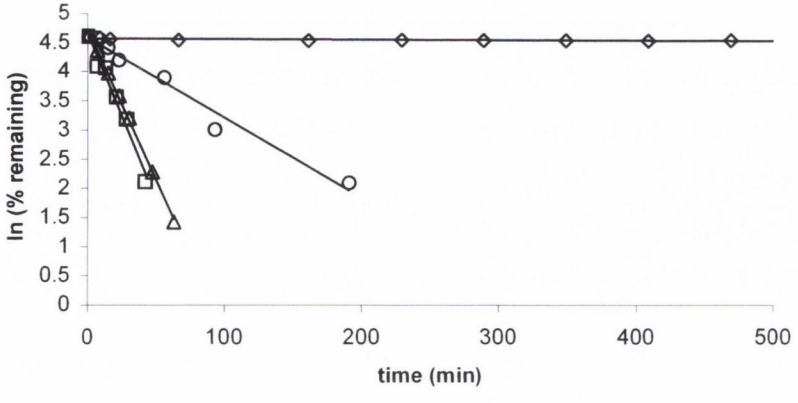


Figure 4. 26: Pseudo-first-order curves for the disappearance of C39

(◊ pH=3.0, □ pH=6.2, Δ pH=7.8, ○ pH=11.6)

As in the case of cyclopentylamine, the indanone derivative of 1-aminoindane (**C01**) is more stable over the full range of pHs than the benzyl acetophenone derivative (**C39,** Figure 4.27). The rates of degradation of this compound are of the same order of magnitude as the rates of degradation of the tertiary amine corresponding to the indanone analogue (**C04**).

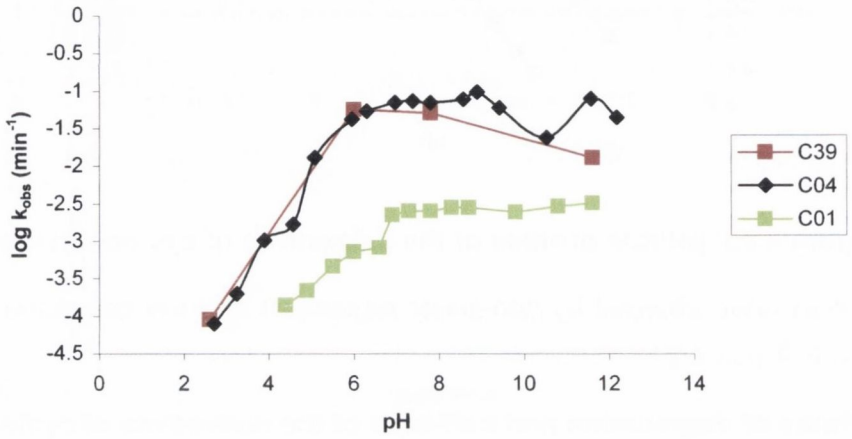


Figure 4.27: pH/rate profiles of the derivatives of 1-aminoindane

The rates of degradation and half-lives at basic pH as determined by non linear regression, are listed in Table 4.4.

Table 4.4: Rates of degradation and half-lives of the derivatives of 1-aminoindane

	k_{obs} (min^{-1})	$t_{1/2}$ (min)
C01	0.0024	288
C04	0.073	9.5
C39	0.054	12.8

For compound **C39, t**he lack of points in the ascending part of the pH/rate profile did not permit a good fit of data to the sigmoidal curve defined by the special case of the Boltzmann equation (**3.9**).

4.4.3.7. Phenylethylamine (C40)

The pH/rate constant profile of compound **C40** was determined in the pH range 2.8-11.5 (Figure 4.28). The compound was found to degrade at a maximal rate at pH=7.4 with a half-life of 5.3 minutes. Phenylethylamine was released concomitantly.

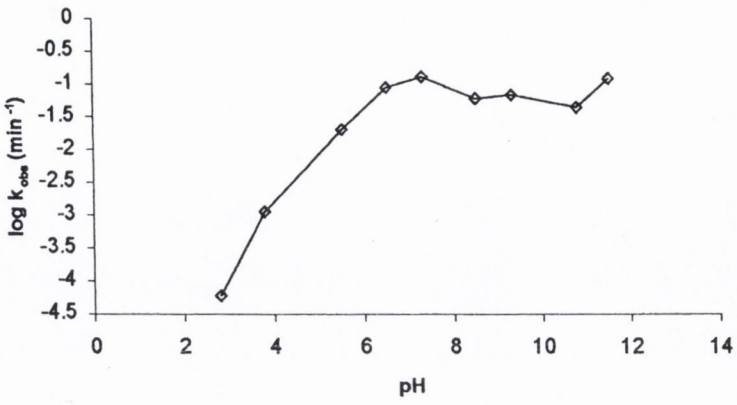


Figure 4.28: pH/rate constant profile of compound C40

Figure 4.29 illustrates the curve obtained by non-linear regression of the data under pH=8.

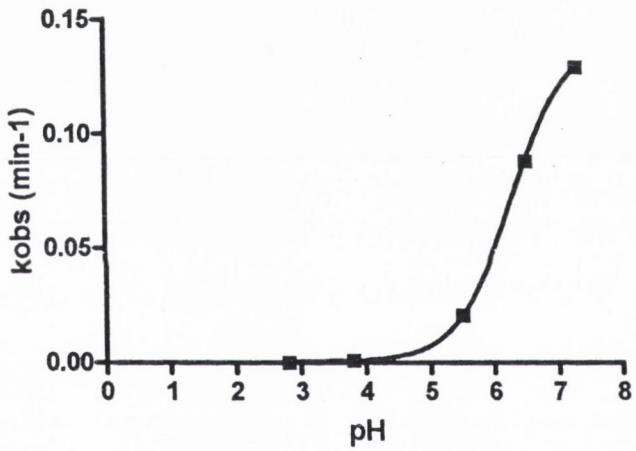


Figure 4.29: Fitting of equation 3.9 to the pH/rate profile of C40

The rates of degradation of this compound and the indanone analogue **C15,** at basic pHs, as afforded by non-linear regression are presented in Table 4.5 evidencing that **C40** degraded much faster than the compound that contains the indanone promoiety.

Table 4.5: Comparison between the rates of degradation and half-lives of the derivatives of phenylethylamine

	k_{obs} (min^{-1})	$t_{1/2}$ (min)
C15	0.0030	231
C40	0.14	4.9

The pK_as of the different compounds can be determined based on the sigmoidal curves fitted to the pH/rate coefficient profile (Table 4.6). Due to the small number of data points available for some compounds, particularly in the ascending portions of the curves, it was

not always possible to estimate the error that affects the fitting parameters. In these cases, the accuracy of the determination may be compromised.

Table 4.6: Parameters of the sigmoid fitting of the pH/rate profile of derivatives of benzyl acetone and benzyl acetophenone

compound	k_1 (min^{-1})	k_2 (min^{-1})	pK_a
C28*	-2e-5	0.21	6.47
C35*	-0.0017	0.12	4.01
C37	0.0026±0.0007	0.22±0.04	6.69±0.24
C38*	3e-5	0.14	6.46
C39*	-0.00092	0.054	4.03
C40	0.00043±0.0012	0.14±0.0025	6.28±0.035

* no error estimate was possible due to limited data

4.5. Determination of the pKas

The pK_as of the test compounds obtained with suitable purity were also determined experimentally as described in the previous chapter for the indanone derivatives (section 3.9). The results are listed in Table 4.7 in conjunction with the pK_as experimentally determined for the free amines and available in the literature.

As mentioned before, the estimates of pK_a based on the pH/rate profiles have an inherent large error due to the limited number of data points used for regression analysis. However, for compounds **C37** and **C40,** for which a larger number of data points were available, the estimates (Table 4.) are in good agreement with the pK_as determined based on mobility (Table 4.7).

Table 4.7: pK_as of the test compounds as determined by capillary electrophoresis

amine	pK_a amine (lit.)	pK_a amine (exp.)	compound	pK_a (exp.)
piperidine	11.2[199]	n.d	**C28**	8.46±.0.14
			C35	7.73±0.10
Propylamine	10.54[170]	n.d.	**C36**	8.35±0.11
hexylamine	10.62[226]	n.d.	**C37**	6.98±0.15
cyclopentylamine	10.7[200]	n.d.	**C38**	7.83±0.10
R-1-aminoindane	9.19-9.24[199]	9.11±0.11	**C39**	5.80±0.38
phenylethylamine	9.83[170]	10.05±0.16	**C40**	6.82±0.11
Desloratadine		n.d.	**C24**	6.97±0.08
			C41	3.65±0.37

During determination of the pK_as, a second ionisation was frequently encountered for the chalcone derivatives at higher pHs (>9). This was evident as in a second sigmoidal curve ranging from zero mobility to mobility towards the anode (negative mobility). This may be due to enolisation of the aminoketone followed by ionisation of the enol group (Figure 4.30).

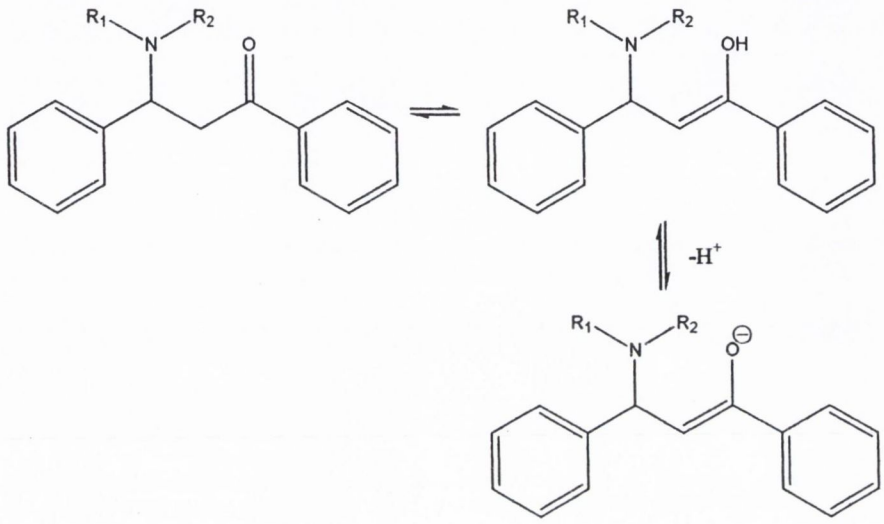


Figure 4.30: Keto-enol equilibria of β-aminoketones

This evidence of facile enolisation further supports the mechanism depicted in Figure 4.18, which consists of an intramolecular transfer of a proton from the OH group to the amine, when the molecule is in the enol form.

4.6. Estimation of log P

The octanol/water partition coefficients of the different molecules were estimated using CS Chemdraw Ultra (Table 4.8).

As in the case of indanone derivatives, a significant increase in lipophilicity is expected for the new ketone prodrug systems. In certain cases the lipophilicities may in fact be larger than is appropriate for oral administration or even impart toxicity to the new compounds.

Table 4.8: Estimations of log P for the test compounds and free amines

amine	Log P (lit.)	Log P (est.)	compound	Log P (est.)
piperidine	0.60*	0.76	**C28**	2.76
			C35	3.90
			C26	2.94
Propylamine	0.28*	0.46	**C29**	2.47
			C36	3.61
hexylamine	2.06*	1.83	**C37**	4.87
			C30	3.72
cyclopentylamine		0.48	**C31**	2.78
			C38	3.92
atenolol		0.50	**C27**	2.09
R-1-aminoindane		1.00	**C32**	3.90
			C39	5.04
2-aminoindane		1.00	**C25**	1.66
phenylethylamine	1.36*	1.48	**C33**	3.66
			C40	4.80
Desloratadine		3.56	**C24**	3.76
			C41	6.86

* from Chemdraw Ultra database

4.7. Conclusions

During synthesis of the test compounds, several difficulties were encountered that reflect problems reported by other authors. The Mannich reaction and Michael addition in organic solution frequently afforded mixtures of compounds that were impossible to separate due to the instability of the compounds.

The most successful reactions were those carried out in so called "solid state" that consisted solely of mixing the amine and the unsaturated ketone together at ambient or sub-ambient temperature in the absence of any solvent. However the reaction only occurs when the amine is a liquid in the base form. Additions to chalcone result exclusively in formation of target compound with degrees of purity suitable for unequivocal NMR identification. Addition to benzalacetone affords mixtures of products if primary amines are used.

Surfactant-assisted aqueous Michael additions offer an alternative for the preparation of compounds but appear to be restricted to the cases where the products are very insoluble in water.

Benzyl acetone and benzyl acetophenone derivatives are particularly unstable towards cleaning up by flash chromatography.

With respect to the stability of the compounds in aqueous solutions, the benzyl acetone and benzyl acetophenone derivatives are more stable in acidic conditions than at physiological pH. Elimination half-lives were in the range 3-13 minutes and were shorter than the half-lives of the corresponding indanone derivatives.

Contrary to what was observed with the indanone derivatives, no evidence of a reverse reaction found for the benzyl acetone and benzyl acetophenone derivatives. This difference could result from the fact that, in indenone, the double bond is necessarily in the *cis* configuration. In chalcone or benzalacetone, configuration can be *cis* or *trans* depending on the conformation prior to elimination. However, *trans* configuration should be preferred and this may hinder the available β position towards addition.

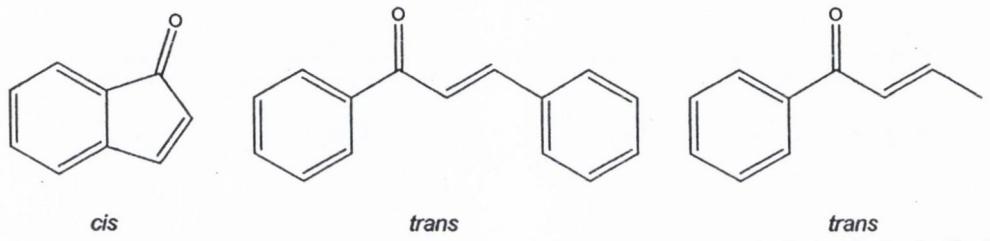


Figure 4.31: Preferable configuration of the double bonds

No conclusions could be drawn about the rate of elimination of amine from the propionate derivatives since the compounds seem to degrade by hydrolysis of the ester group prior to any significant elimination of the amine.

Further tests have to be performed on methyl acetophenones in order to conclude if they can be used as prodrug systems. Information from the literature suggests that these compounds might be overly stable and, therefore might not be a suitable prodrug system. Moreover, vinyl ketones are known mutagenic compounds and this may definitely rule them out as candidates even if in any particular case they degrade at adequate rate.

In terms of their applicability as prodrugs, β-aminoketones derived from conjugated enones, like chalcone or benzalacetone offer an alternative to the originally proposed 2-inden-1-one system. These compounds often degrade at faster rates than indanone derivatives thus enlarging the range of elimination rates attainable by this type of derivatisation.

Solid state and organic solution instability may constitute a limitation to the degree of purity at which the compounds can be obtained. The formation of salts should be attempted to overcome these problems although some authors have referred stability problems also in this form.

Although some compounds eliminate very fast, substitution in the phenyl groups may affect the rates of elimination as observed by other authors[191] and offers a future possibility of further manipulation of the characteristics of the prodrugs.

Secondary effects resulting from release of the promoiety in biological systems should be carefully considered since some chalcone derivatives have been attributed with different biological activities that include anti-oxidant[227], cardioprotective[228], anti-malarial[229,230], anti-tuberculosis[231] and anti-tumour[232,233,234,235] properties.

CHAPTER 5. PRODRUGS FOR L-DOPA AND DOPAMINE

5.1. Introduction

Dopamine and L-dopa have been used for many years in the treatment of cardiovascular, kidney and Parkinson's diseases. However, in all cases, these therapies are far from optimal and many attempts have been made to find prodrugs that are more resistant to first-pass metabolism and allow for more suitable forms of administration and/or fewer side effects.

This chapter provides an overview of the functions of dopamine and L-dopa in the body, their uses in the therapy of the above-mentioned diseases and the limitations of these therapies.

A review is presented of the published attempts to design prodrugs of dopamine, L-dopa and related compounds.

In this work, indanone derivatives of L-dopa ethyl ester and dopamine and also the benzyl acetophenone derivative of dopamine were prepared in order to test, using these drugs, the previously developed concept of β-aminoketones as prodrugs for amines.

The pH/rate constant profiles of these drugs were determined and fitted to an equation that best describes the mechanism of the reaction. The pK_as of the compounds obtained as a parameter of this equation were compared with the pK_as obtained experimentally.

The compounds were also tested for degradation in plasma and for the recovery of intact amine following elimination of the prospective prodrugs.

Some of the compounds were tested for absorption in the everted rat gut model.

5.2. Therapeutic applications of dopamine and L-dopa

5.2.1. A historical overview of dopamine and L-dopa

L-dopa is the natural occurring isomer of the amino acid 3,4-dihydroxy phenylalanine and was first discovered in 1913 in the seedlings of *vicia faba* (broad bean). The racemic mixture had already been synthesised two years previously.

For many years, the view that L-dopa was devoid of biological activity prevailed. It was only in 1938, when dopa decarboxylase (ALAAD) was discovered, that L-dopa's position in the pathway of catecholamine synthesis in the body was postulated[236].

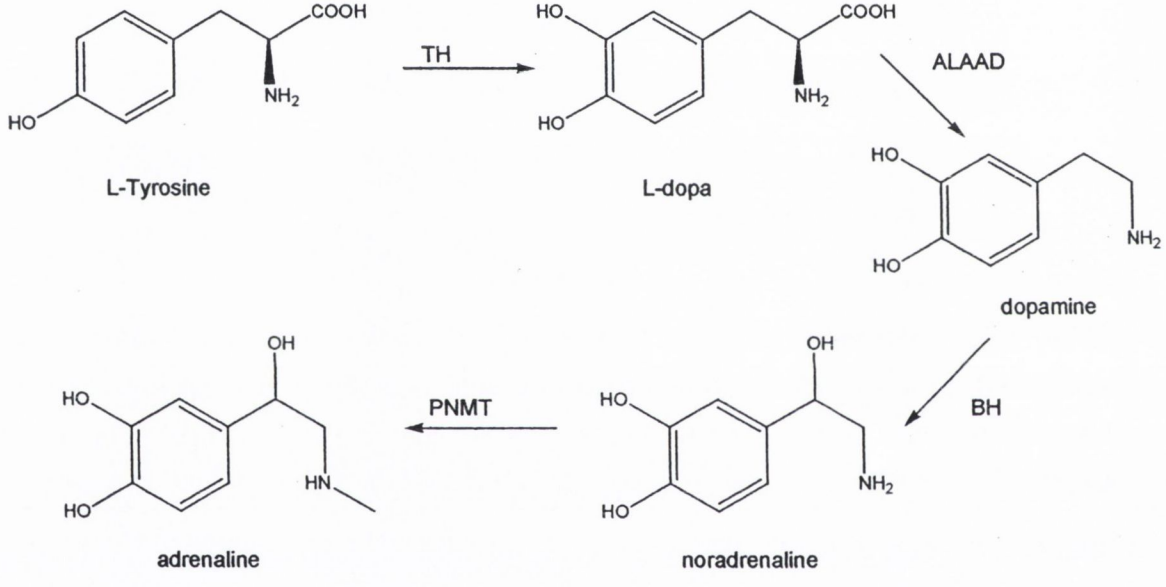


Figure 5.1: Biological pathway for the syntheses of catecholamines

(TH-Tyrosine hydroxylase, ALAAD-Aromatic L-amino acid decarboxylase, BH-β-hydroxylase, PNMT-Phenylethanolamine N-methyl transferase)

In the following decade, the vasoactive characteristics of L-dopa, which had been observed for a long time before, were further investigated and related to the formation of dopamine.

Subsequently, the occurrence of L-dopa in several human tissues, including the brain, was reported and its "catecholamine replenish potential" was extensively studied. Since then the interest in L-dopa and dopamine research has increased substantially[237].

L-dopa was first tested in patients with Parkinson's disease (PD) in 1961[238] and was observed to have a marked beneficial impact in the motor activity of these patients. The subsequent discovery of the striatal localisation of dopamine and its relation to PD, provided a rational basis for the use of L-dopa in this disease. Nevertheless, this view was not widely acknowledged until 1973 (236 and references cited therein). The work devoted

by Arvid Carlson to establishing the role of dopamine as a neurotransmitter, its relative localisation in the brain and its relation to PD, was awarded the Nobel Prize in 2000[239].

Meanwhile, the possibility that L-dopa has a neurotransmitter/modulator role in its own right, has been investigated since 1986[240].

5.2.2. Dopamine, L-dopa and Parkinson's disease

Dopamine is a neurotransmitter, a chemical messenger between nerve cells in the mammalian brain. It is now known that it affects brain processes that control movement, emotional response and ability to experience pleasure or pain. Dopamine is produced from L-dopa by specific nerve cells in the *substantia nigra* of the basal ganglia in the mid- brain.

It is at the points of contact between nerve cells, the synapses, that dopamine is produced and stored in vesicles. When a nerve impulse causes the vesicles to empty, dopamine binds to receptors in the membrane of the receiving cell enabling messages to be carried into the cell. If levels of dopamine are depleted, messages may not be transducted from one neuron to another and motor alterations can be observed.

Parkinson's disease is believed to be caused by a dopamine deficiency in the striatum due to the loss of 70-75% of the dopamine neurons[241]. People with PD suffer increasing motor behaviour impairment that presents initially as milder difficulties with movement and balance and progresses to a complete loss of motor function.

There are several approaches for medicating Parkinson's disease that can be used at different stages of the disease. One therapeutic example is the administration of substances that mimic dopamine: these are dopamine agonists that bind to the dopamine receptors allowing messages to pass in the same manner as dopamine. Dopamine antagonists may also be needed to control dyskinesias (uncontrolled movements) due to over-sensitisation of the dopamine receptors; these drugs bind to the receptors but do not stimulate them preventing over-stimulation by dopamine or its agonists. In milder cases of the disease it is possible to use drugs to assist the nerves in releasing the stored dopamine. Also in the early stages of the disease certain drugs can be used to correct the dopamine/acetylcholine balance and reduce tremor and muscle stiffness. Enzyme inhibitors may be used to help conserve the dopamine in the brain by reducing its metabolism[242].

Dopamine itself cannot be administered to replace the missing resources because it does not pass through the BBB unlike L-dopa, its immediate precursor in the catechol synthesis. In spite of all the alternative therapies and all the research devoted to find superior drugs,

L-dopa remains the most effective treatment for alleviating PD symptomatology[21]. Prior to the era of L-dopa, the excess mortality due to PD was about three times that of the normal population. Studies show that this has been reduced, by at least half, and there is a shifting of incidence to much older groups[243]. Nevertheless, the treatment has certain disadvantages, as some symptoms escape from adequate control and some refractory disabilities may emerge after prolonged treatment[21].

One of the main problems with L-dopa is its low bioavailability. It is absorbed primarily in the small bowel and passes through the BBB by means of a special carrier transport mechanism in competition with neutral large aminoacids[244,245]. L-dopa bioavailability is very sensitive to digestive patterns[244] and consequently to the age of the patients[246] and to their type of diet[16]. Usually, the area under the plasma concentration-time curve of L-dopa is significantly greater in older people[247] due to lower plasma clearance and lower volume of distribution for L-dopa[246]. Diets with high levels of protein reduce L-dopa absorption[16]. Nevertheless, metabolism is one of the main contributing factors for the low levels of L-dopa reaching the brain.

L-dopa relies on aromatic L-amino acid decarboxylase (ALAAD) to be converted to dopamine in the brain. ALAAD is a ubiquitous and non-specific enzyme, with particularly high activity in the stomach, intestinal mucosa, liver and brain[248]. For this reason L-dopa can also be converted to dopamine peripherally. This is undesirable, not only because high levels of dopamine in the periphery can cause side effects, but also because it reduces the amount of L-dopa reaching the brain[16]. In the absence of an ALAAD inhibitor, less than 30% of the drug reaches the systemic circulation[247].

To avoid this, treatment with L-dopa is accompanied by carbidopa or benserazide, which are drugs that block the peripheral decarboxylase. They do not cross the BBB and thus do not affect the release of dopamine inside the brain. By administering these inhibitors, lower doses of L-dopa are necessary and lower side effects are observed. The half-life of L-dopa is increased from about one hour on its own to 1.5-2 hours with carbidopa. At the same time, the daily L-dopa requirements may be decreased by 80%[245].

Several other enzymes metabolise dopamine and L-dopa as depicted in Figure 5.2.

The principal metabolite, in the presence of ALAAD inhibitors, is 3-O-methyldopa (3-OMD) and its production is due to the activity of catechol-O-methyltransferase (COMT). COMT is a non-specific enzyme that catalyses the transfer of a methyl group from S-adenosyl-L-methionine (SAM) to the 3-hydroxyl group of many catechol substrates. It is found in the mucosal layer of the gastrointestinal tract, liver, brain and kidney.

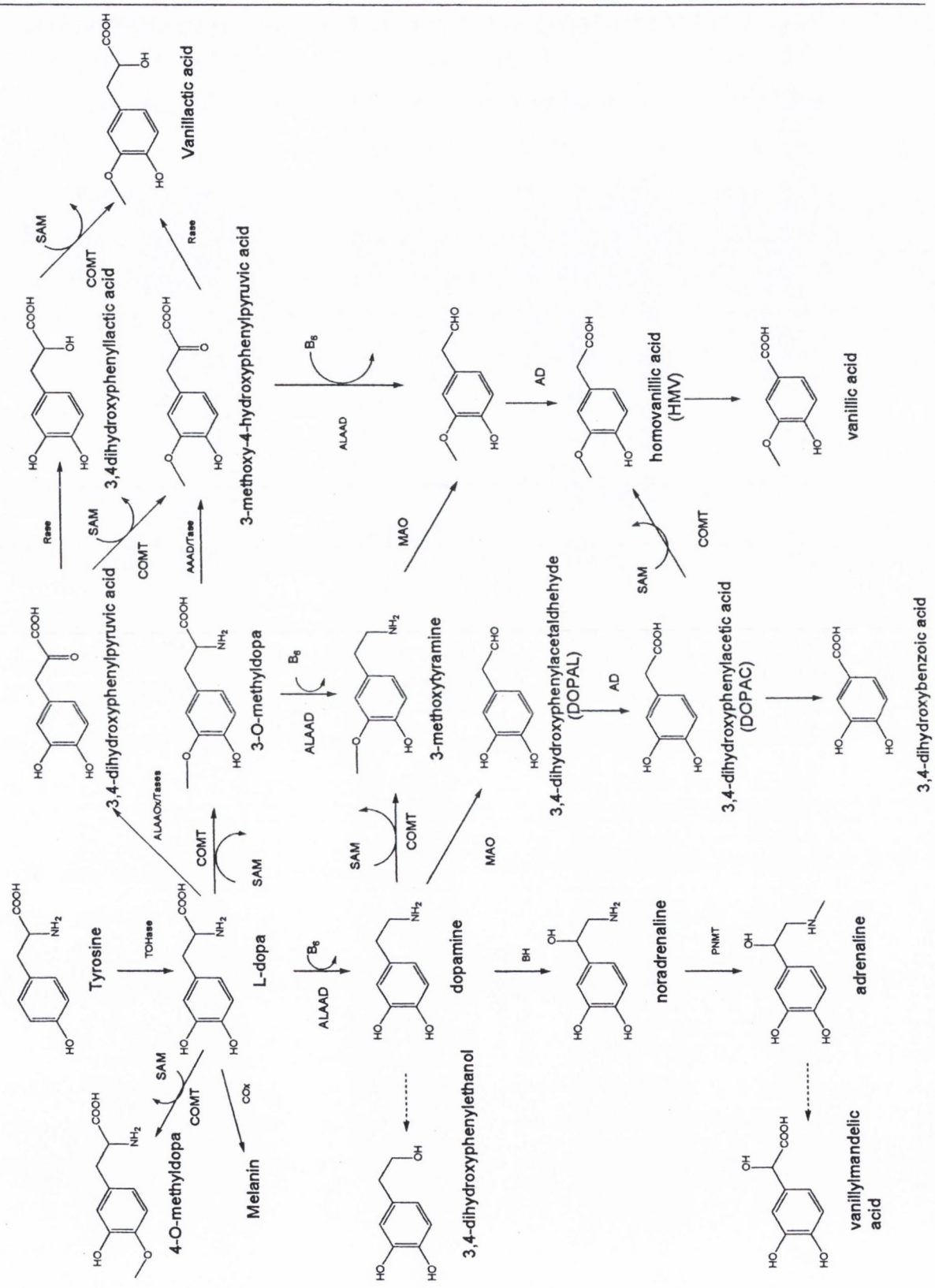


Figure 5.2: Major pathways of L-dopa metabolism. ALAAOx= aromatic L-amino acid oxidase; AD=aldehyde dihydrogenase; βOhase=β-hydroxylase; Cox= catechol oxidase; PEMTase=phenylethanolmethyl transaminase; Rase=reductase; SAM=S-adenosyl-L-methionine; Tase=transaminase; TOHase=tyrosine hydrolase; others as text. Adapted from 248.

Due to COMT activity, only 5-10% of the administered dose of L-dopa reaches the brain. In view of this, the ratio of mean plasma 3-O-methyldopa to L-dopa can be as high as 14:1. The metabolite, which is believed to compete with L-dopa to pass the intestine and the BBB[16], is not known to have antiparkinsoninian activity[248].

Some authors have tried to correlate the presence of toxic levels of dopamine metabolites, particularly metabolites resulting from methoxylation of the catechol group, with the emergence of motor side effects. It was observed that 3-methoxy tyramine and 3,4-dimethoxyphenylethylamine are behaviourally active in rats and interact with the binding sites of dopamine[249].

Monoamino oxidase (MAO), catalyses the oxidative deamination of several amines. It exists in two forms, MAO-A and MAO-B, and the latter preferentially oxidises phenylethylamines and benzylamines. Selegiline inactivates MAO-B and is a commonly used therapy for PD. MAO-B activity accounts for the elimination of about 50% of the administered dose of L-dopa in the form of homovanillic acid (HVA) and 3,4-dihydroxyphenylacetic acid (DOPAC)[248].

Metabolites like 3,4-dihydroxyphenylacetaldehyde (DOPAL) are believed to be responsible for triggering dopamine neuron loss in Parkinson's disease[250]. As a consequence of the disease state, there is enhanced metabolism of dopamine and an increase in the formation of H_2O_2. This leads to the generation of free radicals that further destroy striatal dopaminergic neurons. This phenomenon is termed oxidative stress[251].

It has been very difficult to correlate plasma concentrations of L-dopa with observed response, but it appears that the equilibrium half-life correlates well with the duration of response. The flux of amino acids across the BBB is bi-directional and for this reason the net flux of un-metabolised L-dopa is from the brain to plasma when its plasma concentration falls[16].

In an attempt to lengthen the relatively short duration of action of the established oral L-dopa preparations, alternative formulations and routes of delivery have been investigated. Time "on" seems to be improved by continuous administration[21] such as duodenal infusion and, to a lesser extent, by continuous gastric infusion and controlled release formulations[243,252]. In general, new technologies that deliver constant dopaminergic stimulation seem to, not only eliminate the unpredictable swings in therapeutic efficacy in patients with "on/off" effect, but also have a role in preventing the development of such fluctuations in patients treated chronically with dopaminergic therapies[253].

The controlled release (CR) form of the drug Sinemet (L-dopa+carbidopa) produces more sustained plasma levels of L-dopa but the bioavailability of the drug is about 70% lower.

For these reasons higher doses have to be administered but less frequent dosing is necessary[254].

Intranasal or transdermal administration may also improve not only bioavailability but also the overall pharmacokinetics of L-dopa, by avoiding passage through the gastrointestinal tract and improved absorption[16].

Long-term treatment with L-dopa causes severe side effects. After a few years of stable response to L-dopa, there tends to be decreased control of parkinsonian symptoms. The patient begins to experience end-of-dose deterioration (wearing-off) a few hours following each dose. As a consequence of the disease, there is a diminished amount of dopamine available and the organism tries to compensate by "making" the receptors more sensitive to dopamine. When L-dopa is administered there is a peak concentration of dopamine available after each dose, that can cause over-stimulation of the receptors with consequent severe and rapid fluctuations in mobility (dyskinesias)[252]. There are also diurnally random episodes of the so-called "on-off" phenomena (which may or may not be related to doses and times of administration[255]), episodes of freezing of movement, increased fatigue and psychological alterations[243].

Although "wearing-off" responses disappear promptly upon stabilisation of circulating L-dopa levels, fluctuations of the "on-off" type are more difficult to control; nevertheless, with round-the-clock L-dopa administration for about nine days, the effect decreases in about 50% of cases[256] and there is evidence that there is a threshold plasma concentration of L-dopa associated with the switch "on" or "off" effect[244].

Keeping in mind the complications that may emerge following long term treatment with L-dopa, some people think that its initiation should be deferred to more advanced stages of the illness, in favour of dopamine agonist monotherapy, especially in young patients[257,258]. Although several controlled studies, comparing L-dopa and dopamine agonists as initial treatment, have attempted to answer the question of whether delaying L-dopa therapy can reduce the occurrence of motor complications, there is no clear answer as one has to balance the beneficial effects versus the disadvantages of the treatment[21].

Future therapies of the disease will focus on control of oxidative stress and other neuroprotective treatments, surgical approaches, including transplantation of dopaminergic embryonic tissue, and gene therapy[257].

5.2.3. Dopamine, L-dopa and cardiovascular and renal diseases

Peripheral dopamine receptors are important in the control of hypertension. They are subdivided in two distinct subtypes, DA-1 which are located on vascular smooth muscle, in renal proximal tubules and juxtaglomerular cells and DA-2 which are located on the postganglionic sympathetic nerve terminals. Activation of DA-1 leads to vasodilation, inhibition of tubular transport and renin release whereas DA-2 activation inhibits norepinephrine release. A defect in peripheral dopamine receptor function has been proposed to be involved in the pathogenesis of hypertension[259].

Dopamine has widespread effects and cannot be orally administered mainly due to presystemic inactivation by sulfate and glucuronide conjugation[260]. Intravenous infusion is the most common form of administration in intensive care units. In spite of some controversy in respect of the real beneficial effects of therapy with low doses[261], dopamine is believed to promote vasodilatation and hypotension. At higher doses the opposite effect is observed since it increases cardiac contractility and output through stimulation of β_1-adrenoceptors[259]. L-dopa, on the other hand, also has frequent adverse effects as mentioned before.

As a consequence, other prodrugs of dopamine and dopaminergic drugs have been developed in an effort to achieve improved bioavailability and reduced β-adrenergic vasoconstrictor action[259,260].

5.3. Published strategies for the development of prodrugs for dopamine, L-dopa and related compounds

The problems associated with long-term therapy of Parkinson's disease with L-dopa and also the limitations of dopamine for cardiovascular treatments, lead to the search for prodrugs (for L-dopa or dopamine) with better performance characteristics.

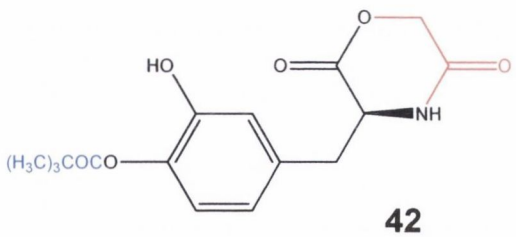
In the case of L-dopa, Bodor *et al*[262], have protected, individually and in combination, the three sensitive centres in the molecule: the catechol system, the amino group and the carboxyl function. They focused on reducing the pre-systemic metabolism of L-dopa and increasing its bioavailability while maintaining or improving its properties in terms of water and lipid solubility. Most attempts lead to an increase in the maximum concentration of L-dopa found in the blood following oral administration, with no significant increase in the maximum concentration of dopamine. This increased ratio of L-dopa/dopamine is considered advantageous since it reveals in a decrease in peripheral metabolism.

5.3.1. Protection of the catechol group

A common approach to the protection of the catechol group involved the formation of esters such as acetyl and pivalyl[262], benzoyl[263], isopropyl and phosphatyl[264].

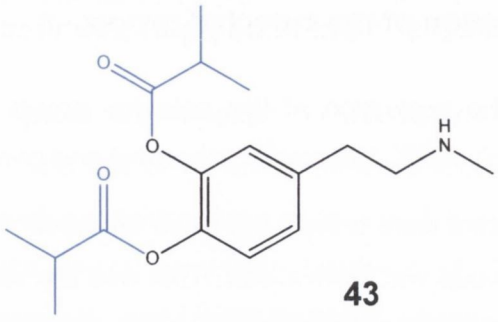
For the most part, the diesters were tested, but there are several examples in the literature where only one hydroxyl group was derivatised. This was the case of the single ester L-3-(3-hydroxy-4-pivaloyloxyphenyl)alanine[265,266,267] which received considerable attention probably due to the large increase in L-dopa bioavailability after administration of the compound (7.6 fold)[265]. This prodrug of L-dopa seems to have a prolonged duration of action (2.3 fold in rats when compared to L-dopa) and reduced severity of peak-dose dyskinesias. Higher doses have to be administered to humans to achieve the same effect, although bioavailability was increased in rats (1.4 times). However, the compound isomerises in solution by migration of the pivaloyl group to the 3-position and this isomerisation may impair the use of the prodrug[268].

Further derivatisation of this compound was carried out at the amino and carboxylic groups using a promoiety sensitive to physiological pHs. The new compound L-3-(3-hydroxy-4-pivaloyloxybenzyl)-2,5-diketomorpholine (**42**) is stable in acidic conditions, passing unhydrolysed through the stomach after oral administration. At pH 7.4 the prodrug is slowly hydrolysed ($t_{1/2}$=1.5 h) allowing it to be absorbed in the intestine before releasing L-3-(3-hydroxy-4-pivaloyloxyphenyl)-alanine, which is subsequently enzymatically hydrolysed to L-dopa[269].



42

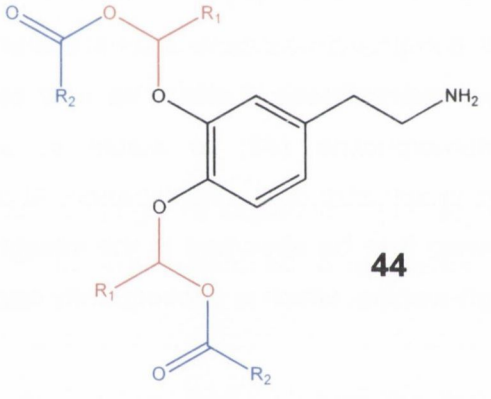
Ibopamine (**43**) was developed as an oral prodrug of epinine (N-methyl dopamine) which is a slightly more potent peripheral dopamine agonist. There is a reported lack of effect of ibopamine on renal function in healthy patients[270]. Others state that Ibopamine is devoid of central effects when administered peripherally but its peripheral pharmacological properties are qualitatively similar to those of dopamine[259,271].



43

Nevertheless, and in spite of the fact that the early clinical studies with ibopamine, as a diuretic in heart failure, were favourable[272], there is evidence of increased mortality and many studies have been carried out to evaluate this data[273].

Another approach to the protection of the catechol group of dopamine was the preparation of acyloxyalkyl ethers (**44**)[274].



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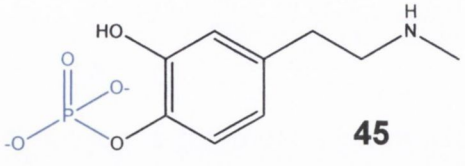
The 4-O-phosphate of N-methyl dopamine (**45**)[260,275] was developed as a prodrug of N-methyl dopamine, designed for preferential delivery to the kidney[271].



45

5.3.2. Esters as a form of derivatisation of the carboxylic group

As expected, esters have also been used to protect the carboxyl group. Methyldopa for example benefited from the preparation of the pivaloyloxyethyl ester in terms of absorption following oral administration[276].

Short chain alkyl esters have been widely studied, since the late 1980's, with promising results.

Response fluctuations to L-dopa may be the result of pharmacokinetic factors like delayed gastric emptying. Bypassing the stomach, by delivering levodopa parenterally, may be an optimal solution although it requires a water soluble form of the drug[277]. Most of the short chain alkyl esters are highly water soluble which allows for different modes of administration such as subcutaneous and intramuscular injections, which can be advantageous in the rapid therapy of patients in "off" situations[278,279].

Initial tests with various esters did not reveal any marked behavioural improvement in comparison to L-dopa when administered intraperitoneally or orally in rats and mice although, in some cases a slight increase in activity was observed[280,281]. However, results from tests with rats are difficult to extrapolate to humans, since rats have carboxylesterase activity in plasma that does not exist in humans[282].

Tests with these compounds were further extended and they were also evaluated for rectal[283] nasal[284] and transdermal[285] administration.

L-dopa methyl ester (LDME) and L-dopa ethyl ester (LDEE, etilevodopa) have been clinically evaluated. Liquid forms of the LDME and LDEE were administered orally to groups of patients respectively as a single dose[286,287] or over a period of 4 weeks to 6 month[288,289]. The results from the trial with LDEE showed a decrease in the percentage of "no-on" episodes and latency to "on"[289]. With an oral solution of LDME, there was a significantly more rapid reversal of "off" periods[287,288].

Since LDEE is water soluble, it can be administered parenterally thus avoiding the gastrointestinal tract, but even when administered orally, the compound demonstrates more rapid absorption and reduced latency to turning on[278]. Bioavailability was not significantly increased in humans (although, apparently, there was an increase of bioavailability when esters were administered to rats and dogs).

In the case of LDME, the magnitude of clinical response obtained with a single bolus injection was the same for L-dopa and the ester, although the duration of action of the ester was shorter and higher[290]. However, LDME administered concomitantly with a slow release formulation of L-dopa might provide a useful balance between rapid onset and extended duration of action[291]. With direct administration of LDME to the intestine, by infusion pump, higher infusion rates were necessary in comparison to L-dopa[292].

Despite the initially promising results, phase II trials with LDEE were cancelled in the first quarter of 2003.

5.3.3. Derivatisation of the amino group

Dopamine has been derivatised with amino acids but disappointing results were obtained with these compounds following oral administration[260].

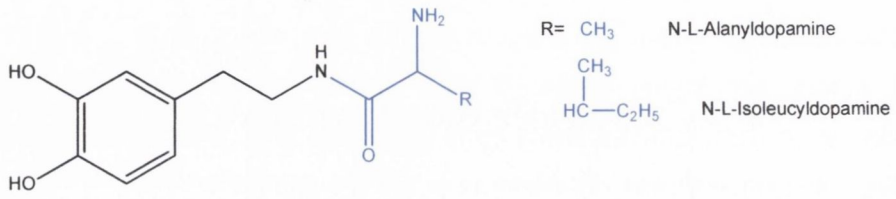
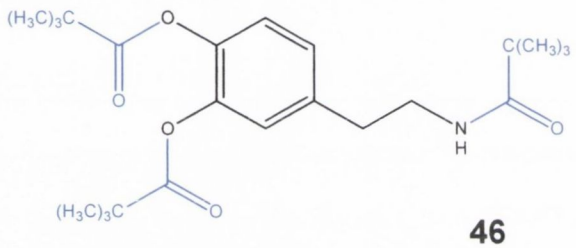


Figure 5.3: Amino acid derivatives of dopamine

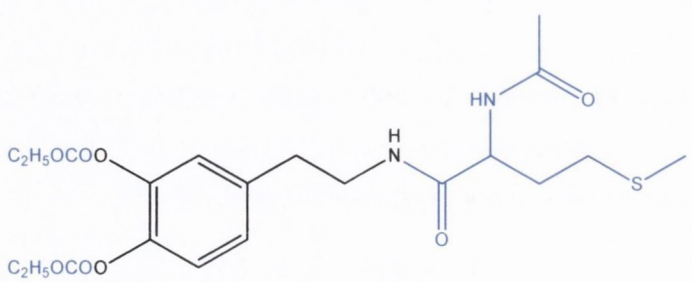
A tripivaloyl form of dopamine (**46**), where both the catechol and the amine group are protected, has been evaluated.



46

Tests in rats showed that this prodrug was able to cross the BBB and induce chewing behaviour comparable to that obtained with intracereboventricular injection of dopamine. It also produced a long lasting effect that was attributed to a slow release of dopamine due to some steric resistance to enzyme hydrolysis at the amide bond. The peripheral release of dopamine was thought to be of no biological significance, since no α-adrenergic effect was observed[293].

Docarpamine, [N-(N-acetyl-L-methionyl)-O,O-*bis*(ethoxycarbonyl)dopamine)] (**47**) is a successful prodrug of dopamine that can be orally administered and is used in renal and cardiovascular pathologies[294,295]. The prodrug has been commercialised in Japan since 1994 in the form of granules as a prescription-only drug, under the brand name of Tanadopa® (Tanabe Seiyako Co., LTD).



47

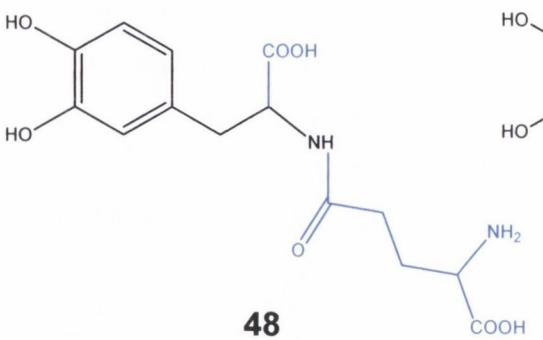
It has been developed in an attempt to overcome inactivation of dopamine by sulfotransferase, MAO and COMT in the intestine wall and liver[296]. The compound is well absorbed in the digestive organs after oral administration, has weak vasodilatory effects on its own right[297], has no effect in CNS even at high doses[296] and is easily hydrolysed to dopamine[294].

Oral tests in rats and dogs produced lower peak concentrations of dopamine on administration of docarpamine but gave areas under the curve values of free dopamine that were 4-6 times higher than those achieved with equimolar doses of dopamine[298].

First pass metabolism of the compound in dogs was investigated and it showed that the major pathway of degradation in the small intestine is by catechol ester hydrolysis. In the liver, ester and amido-linkage hydrolysis, as well as dopamine conjugation, were the major metabolic pathways[299]. Preliminary clinical trials suggest that docarpamine may be useful in patients with low cardiac output syndrome following cardiac surgery and in refractory cirrhotic ascites[272].

As mentioned before, γ-glutamyl derivatives have been prepared in an attempt to produce prodrugs with beneficial cardiac and renal effects.

N-derivatives of L-dopa and dopamine have been prepared and displayed significant renal selectivity thus avoiding widespread peripheral effects[272]. When the double prodrug L-γ-glutamyl-L-dopa (Gludopa) (**48**) is administered, L-dopa is released and decarboxylated in the kidney where ALAAD is also abundant. Amongst other dopamine prodrugs, Gludopa was the only one that did not produce hyperglycaemia at doses that are known to increase renal plasma flow in the rat[300].



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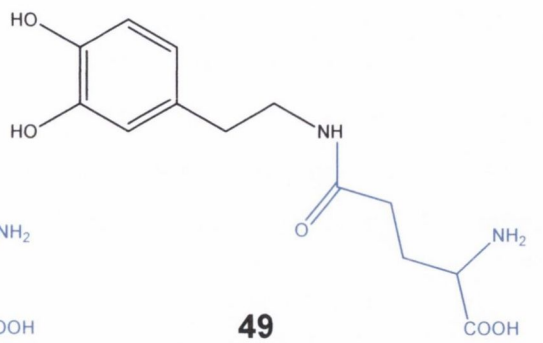


49

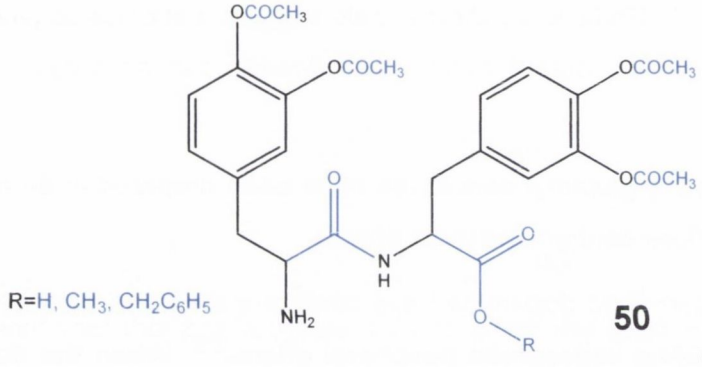
In the case of L-γ-glutamy-L-dopamine (**49**), the prodrug is cleaved slowly in the proximal tubules producing the desired effect and excess dopamine is metabolised and excreted without returning to the systemic circulation[44,301,302].

5.3.4. Peptides and macromolecular prodrugs

As well as glutamyl derivatives, several other compounds were prepared making use of the peptide bond.

Peptides for example, have been used for protection of the amino and the carboxyl functions. Both di and tripeptides were tested, with and without[262,303] masking of terminal amino, carboxyl and catechol functions with N-phthaloyl, carbobenzoxy and formyl groups[304]. Some compounds were more and others were less effective than L-dopa but no structure activity relationship was identified[304].

Interesting examples include the dipeptides of L-dopa (**50**), which demonstrated highly favourable L-dopa/dopamine ratios[262].



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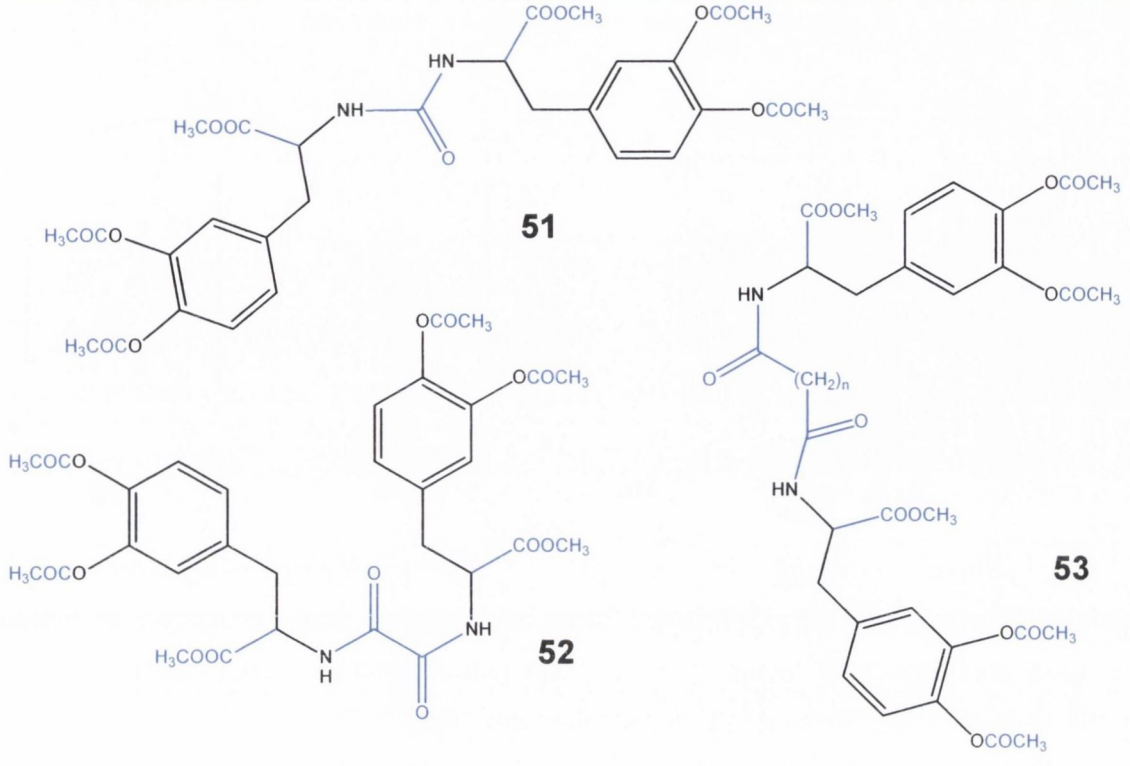
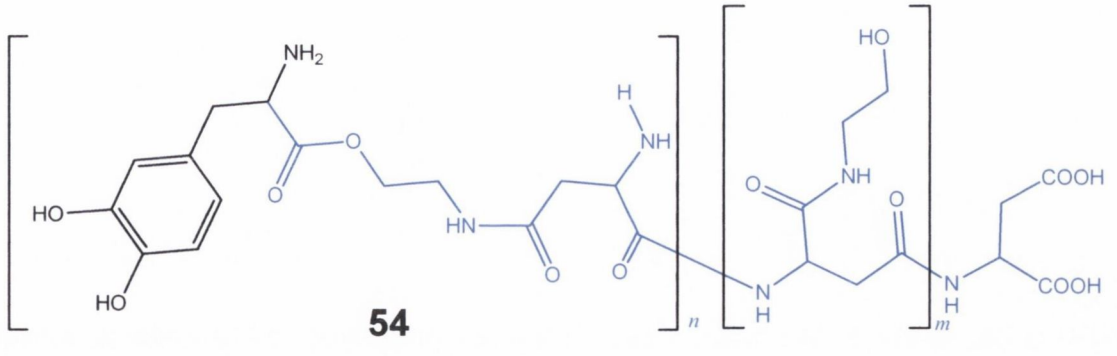
Peptides and aminoacids exploit the intestinal peptide transporter to enhance absorption and peptidases to promote cleavage. Inhibition of the uptake of the test compounds (p-Glu-L-dopa-Pro[305] D-*p*-hydroxyphenylglycine-L-Pro-L-dopa[306] and L-dopa-D-phenylglycine[307]) on the brush border membrane vesicle in the presence of other peptides, demonstrated the active transport of the compounds. D-*p*-hydroxyphenylglycine-L-Pro-L-dopa was well absorbed and exhibited an anti-Parkinsonism effect[306]. L-dopa-D-phenylglycine demonstrated improved absorption (approx. 50 fold in terms of molar ratio) in comparison to L-dopa[307]. D-phenyl glycine was also tested as a peptide mimetic carrier system for α-methyldopa. Some of the prodrugs tested had better absorption profiles than the original drug[308].

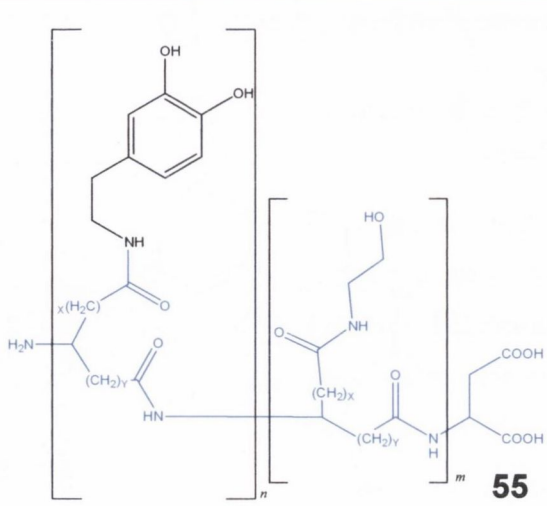
Recently, other dimeric forms of L-dopa (**51-53**) have received some attention, demonstrating lipophilicities suitable for oral administration, good *in vitro* stability and enzymatic cleavage in 80% human plasma with slow release of L-dopa[309]. Bioavailability tests have not yet been reported.



Still in the field of macromolecular prodrugs, a polymeric α,β-poly(N-hydroxyethyl)-DL-aspartamide (PHEA) adduct with L-dopa (PHEA-L-dopa) (**54**), linked by an ester bond at the carboxylic function, was prepared and found to have an *in vitro* half-life of 10.9h[310]. An encapsulation of this system in alginate-chitosan microspheres was also tested to provide protection from the surrounding medium while delivering the prodrug[311].



Similar systems were applied directly to dopamine, not only with α,β-poly(N-hydroxyethyl)-DL-aspartamide (PHEA-Dopamine, **55**) but also with a styrene-maleic anhydride copolymer (SMA-Dopamine, **56**). The percentage of dopamine in each compound is 14.3 and 42.8% respectively. In both cases the amide linkage to dopamine can be hydrolytically or enzymatically cleaved but rates of cleavage at physiologic pH are lower than at other pHs[312]. No data, evaluating the kinetics in plasma or *in vivo*, was provided.



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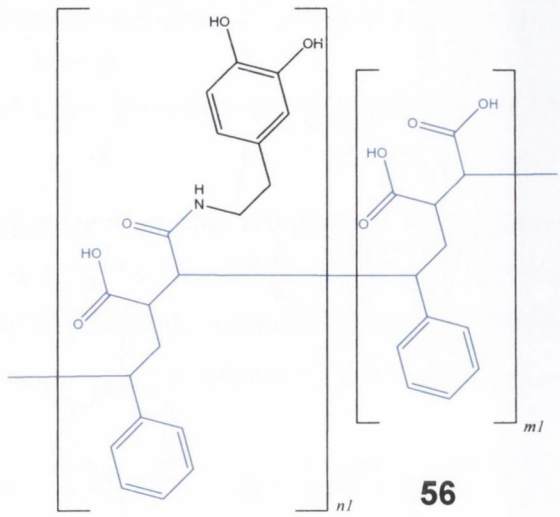
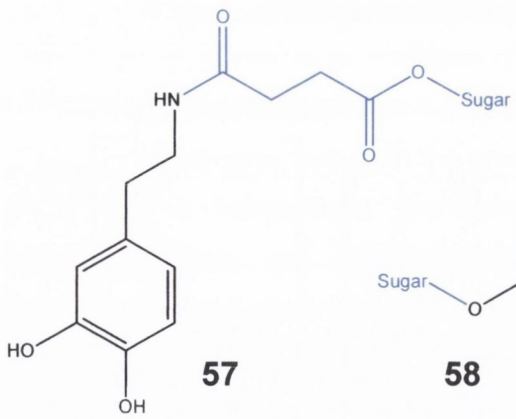


56

A lymphotropic prodrug of L-dopa, 1,3-dihexadecanoyl-2-[(S)-2-amino-3-(3,4-dihydroxyphenyl)propanoyl] propane-1,2,3-triol administered orally, produced an increase of L-dopa and dopamine levels in mice brain (when compared to L-dopa), for longer periods and with more favourable L-dopa/dopamine ratios[313].

Other specialised transport systems have also been targeted such as the glucose carrier GLTU-1, which is located in the BBB. With this objective, several glycosyl derivatives of dopamine have been prepared (**57-59**).



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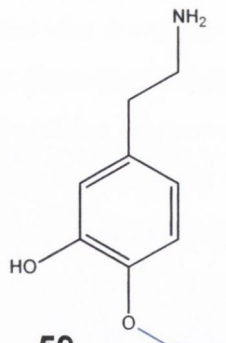


59

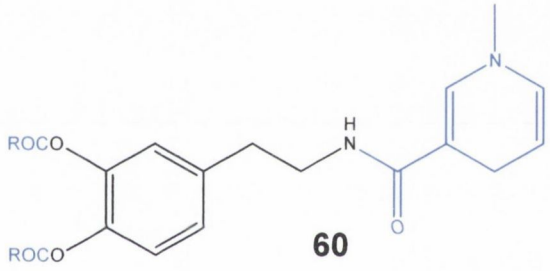
Following transport into the brain, these derivatives were expected to undergo activation through the successive action of esterases and proteases or amidases in the case of the compound derivatised at the amino group, and by the action of glycosidases when the catechol group is derivatised. Nevertheless, these compounds failed to demonstrate any *in vivo* activity when tested in reserpinized mice. This lack of activity was attributed to a possible inability of the compounds to cross the BBB and also to the slow conversion to dopamine observed in *in vitro* tests[314].

Other authors linked dopamine and L-dopa to glucose and galactose through a succinyl linker and observed activity on models of Parkinson's disease with minimal vascular effects[315].

5.3.5. Redox systems

Regarding the protection of the amino group and targeting the delivery of dopamine to the brain, the aforementioned dihydropyridine redox system[316,317] (page 21) and corresponding dimer systems[293] were tested.

The dihydropyridine system was attached to the amino function while the catechol system was protected by acylation (**60**). After administration, the pyridinium precursor is "locked in the brain" while it is quickly eliminated from the general circulatory system, resulting in brain-specific delivery.



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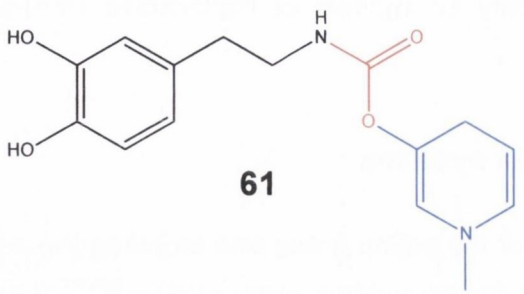
No increase in rat-brain dopamine levels was observed following intravenous administration of the dyhydropyridine derivative (with the catechol group protected as the corresponding dipivalyl ester). This was believed to be due to the facile metabolism and elimination of the dopamine formed, preventing measurable accumulation. However, there was a reduction in prolactin serum concentration, consistent with the slow and persistent release of dopamine[316]. An increase of about 20% of dopamine (in comparison with controls) in the striatum was observed after administration of the prodrug, if endogenous synthesis was inhibited[318]. Nevertheless the concentrations achieved were not sufficient to induce the stereotypic behaviour characteristic of intense dopaminergic stimulation[319].

The system has also been modified to include an activated carbamate ester (**61**). The dihydronicotinate moiety was attached to the amino group in dopamine by acylation with chloroethyl chloroformate followed by condensation with sodium nicotinate. The product was then N-ackylated at the pyridine ring and reduced to the corresponding 1,4 dihydropyridine derivative. The compound is readily converted to the quaternary salt *in vitro*. There was no evidence of sustained levels of the prodrug in the rat brain but there appeared to be some change in the locomotor activity of the animals, which could be due to altered central dopamine neuronal activity[320].



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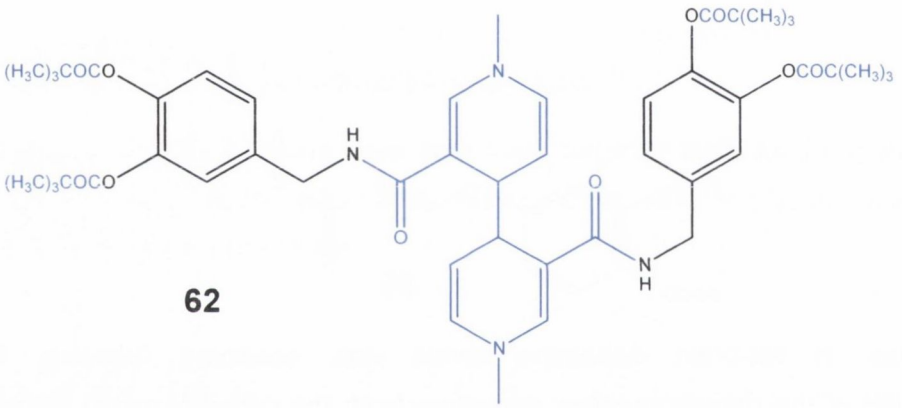
A dimeric form (**62**) of the original dihydropyridine⇄pyridinium system, as applied to the dipivaloyl ester of dopamine, is thought to be more stable in solid state than the monomeric form but the pharmacological characteristics are similar[293].



62

The major drawback of dihydropyridine systems is their facile oxidation in solid state and in solution, which makes formulation difficult. This lead to attempts to find more stable compounds using the same principle. One such system involved an open-ring thiazolium precursor that converts *in vivo* to the ring-closed salt (Figure 5.4). It consists of a thiamine disulfide that has been used clinically as a fat-soluble precursor of thiamine. The concept has been tested with O,O-dipivaloyl-L-dopa and proved to facilitate the delivery and retention of L-dopa and dopamine in the brain[69].

Despite these many attempts to produce prodrugs for dopamine and L-dopa, few of the compounds reached clinical trials and even less are actually commercialised. Docarpamine is a notable example of success on the treatment of cardiovascular diseases, but L-dopa remains the preferential treatment for Parkinson's disease.

In the future, surgery and genetic therapies will probably provide the cure of these diseases. Meanwhile, finding an adequate prodrug to ameliorate the symptoms and provide a better quality of life for the patients is still an important goal.

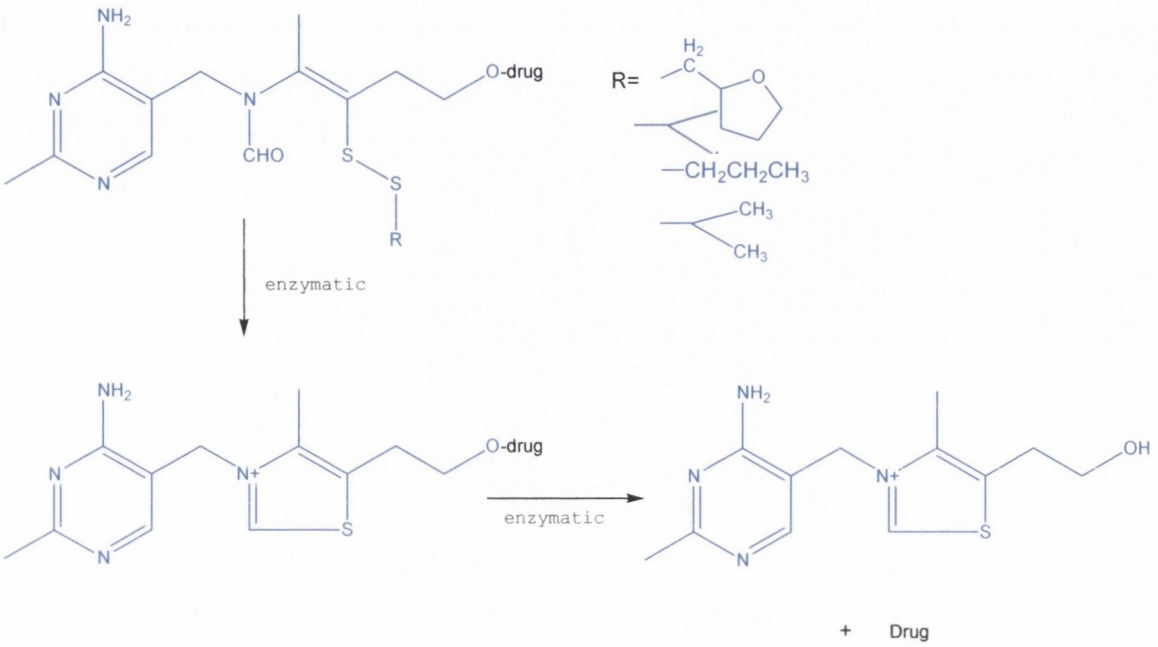


Figure 5.4: Thiazolium redox delivery system

This thesis will present another type of derivative of these drugs with potential to improve delivery to the systemic circulation and to the site of action.

5.4. β-Aminoketone prodrugs for dopamine and L-dopa

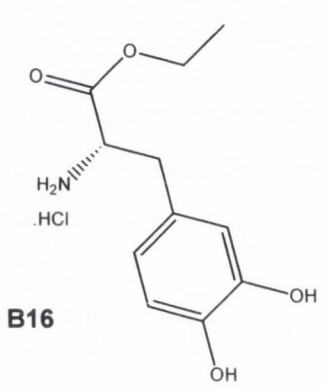
Decarboxylation of L-dopa with consequent peripheral release of dopamine, followed by metabolism to DOPAL by MAO, constitute some of the problems of delivery of dopamine to the brain and are responsible for serious side effects in the treatment of Parkinson's disease. Moreover, despite the promising docarpamine therapy, no optimal solution seems to have been found for oral administration of dopamine in cardiovascular diseases.

For these reasons, the development of prodrugs, which afford protection of the amino group in dopamine and/or L-dopa using derivatives that are resistant to first pass metabolism in the stomach and/or can pass the BBB, is desirable.

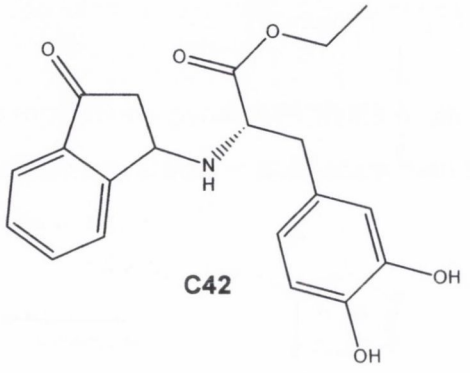
In this context, the aminoketone prodrug systems that were developed in the previous chapters were tested for dopamine and L-dopa. The results of these tests are described in the following sections.

5.4.1. Synthesis of the test compounds

At the time when this work was initiated, etilevodopa (**B16**, ethyl ester of L-dopa) was being tested as a double prodrug of dopamine and the results were very promising. For this reason it seemed natural to produce a prodrug which would afford protection of the amino group even after metabolism of the ester. This prompted the synthesis of compound **C42**.



B16



C42

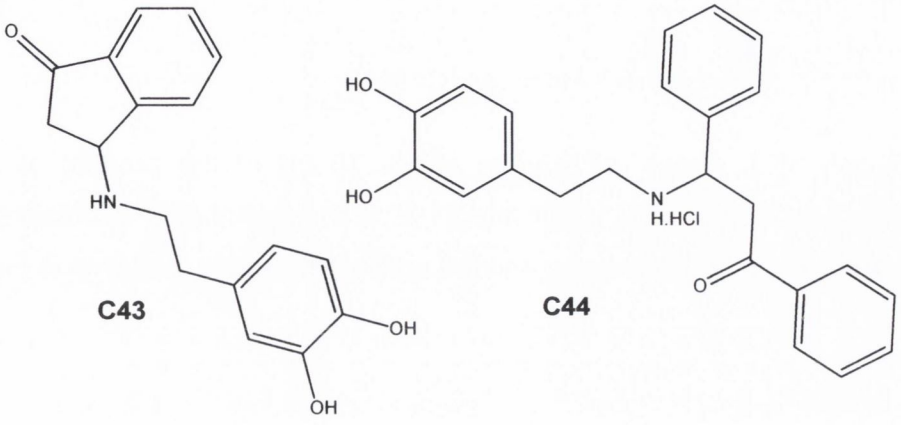
However, early in 2003, the clinical trials were stopped since no important benefits were observed in comparison with L-dopa. Nevertheless, the rational for compound **C42** can still be considered in terms of a sustained release formulation of etilevodopa and consequently L-dopa and dopamine, before or after passage through the BBB.

The ethyl ester of L-dopa was prepared from L-dopa in ethanol at -40°C in the presence of thionyl chloride. **C42**, was then synthesised by substitution of the bromine of 3-bromoidanone in the usual way.

If the increase in lipophilicities, imparted by aminoketone derivatives, affords transport through the BBB, the use of L-dopa might not be necessary and direct derivatisation of dopamine probably makes more sense. On the other hand, even if the compounds are not lipophilic enough to pass the BBB, the prodrugs may be useful for non-nervous system therapies. With this in mind, compounds **C43** and **C44** were prepared as single aminoketone prodrugs of dopamine.

Compound **C43** was prepared by reaction of dopamine with 3-bromoindanone as previously described for other aminoindanones.

The convenient method of Michael addition to chalcone in solventless conditions could not be applied to dopamine for the synthesis of compound **C44** since the hydrochloride salt is the commercial form of dopamine available. Surfactant assisted reaction in aqueous solution did not progress probably due to the good solubility of dopamine in water.



After a few attempts in different solvents with frustrating results, the reaction proved successful using dimethylformamide in the presence of triethylamine and a large excess of the enone. The reaction was monitored by capillary electrophoresis until all amine was consumed. After this, the mixture was diluted with water containing one equivalent of HCl and the excess enone was removed by extraction with DCM.

The aqueous phase, which contained the target compound, was evaporated to dryness and the residue was washed with hexane:ethanol (50:50). During this procedure some deamination was observed and the final product was isolated with small levels of contamination by the free amine (about 3% in peak area as observed by CE).

5.4.2. Elimination of the amine from the prospective prodrugs

The pH/rate profiles of the three prospective prodrugs of dopamine were determined by CE in the range of pH 0.7-11.3 and I=0.154. There was no evidence of establishment of equilibrium as all compounds completely degraded with profiles consistent with pseudo-first-order kinetics. The small increase in the peaks of dopamine or etilevodopa was not consistent with degradation exclusively by elimination of the free amines. In the case of **C42**, this could be due to hydrolysis of the ester, with consequent formation of L-dopa, which would not be detected by capillary electrophoresis (this would not compromise the performance of the prodrug). However, in the case of the single prodrugs of dopamine, another pathway of degradation could potentially reduce the availability of the drug.

A HPLC method was developed and validated for the determination of dopamine recovered from degradation of compounds **C43** and **C44**.

5.4.2.1. Determination of the pH rate profiles by CE

a) 3-Dopamine-1-indanone (C43)

A stock solution of 1 mg/ml (ACN:buffer pH=3, 75:25) of the product of reaction of dopamine with 3-bromoindanone was diluted 1:10 in the test buffers to obtain solutions of 0.35 mM. It was tested for degradation and the profile obtained is displayed in Figure 5.5.

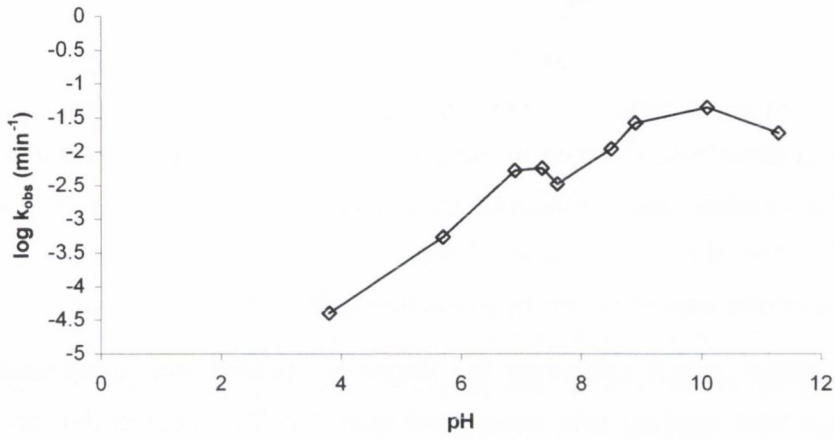


Figure 5.5: pH/rate profile of C43

The compound was stable in the pH range 0.7-2 but degraded at higher pH 's with profiles consistent with pseudo-first order kinetics.

The data for pH under 7.4, was fitted to equation **3.9** and afforded the plot presented in Figure 5.6.

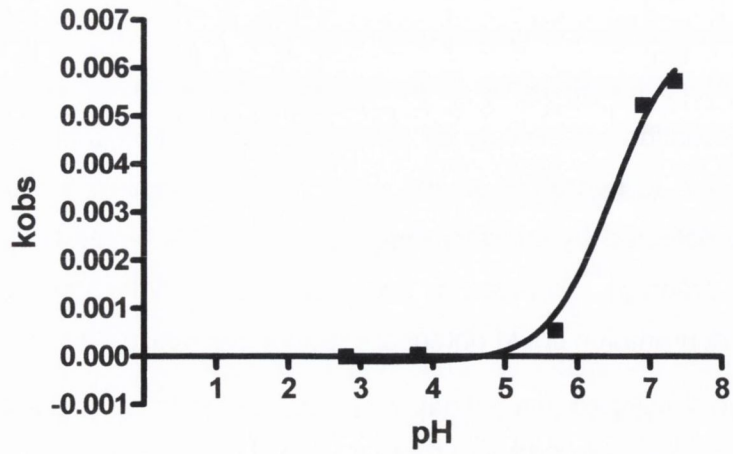


Figure 5.6: Fitting of equation 3.9 to the pH/rate profile of C43

The degradation rate at physiological pH afforded by this fitting was 0.0067 min^{-1}, which corresponds to a half-life of 103 minutes. Despite the fact that the half-life continues to decrease at higher pH, there is evidence that this is due to another degradation reaction at high pH since the detected concentration of dopamine is much smaller (Figure 5.7). This may be due to the subsequent degradation of dopamine. The highest concentration of dopamine was detected at pH=6.9.

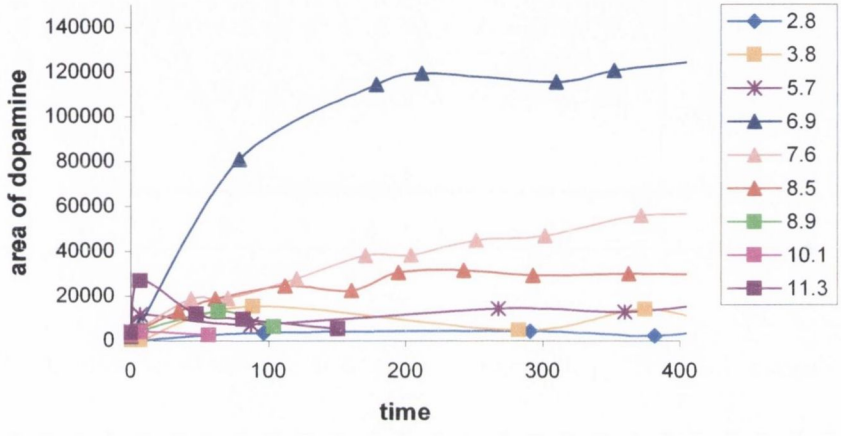


Figure 5.7: Areas of dopamine detected from elimination of C43 at different pH

b) Dopamine-benzyl acetophenone (C44)

The pH/rate constant profile of compound **C44** is depicted in Figure 5.8. As in the case of compound **C43**, the rate of degradation of **C44** at high pH did not result in an equivalent increase in the concentration of dopamine.

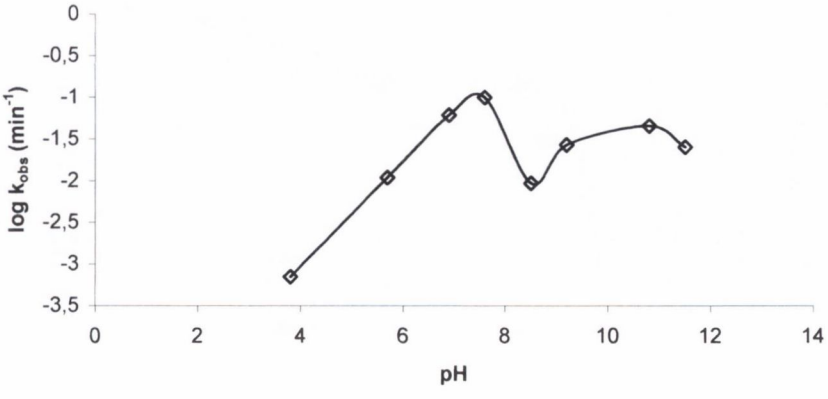


Figure 5.8: pH/rate profile of C44

The data in the pH range 0.5-7.2 was fitted by non-linear regression to a sigmoidal curve as depicted in Figure 5.9.

This fitting afforded an estimation of the half-life of the compound at physiological pH, which was 5.9 minutes.

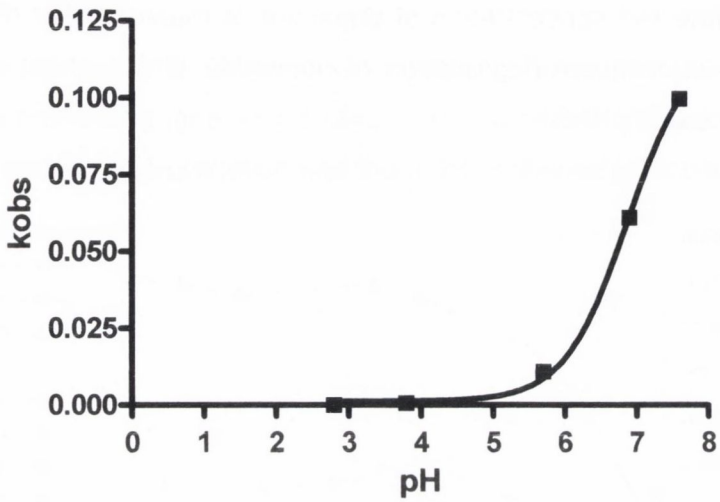


Figure 5.9: Fitting of equation 3.9 to the pH/rate profile of C44

c) L-Dopa ethyl ester indanone (C42)

Electrophoregrams of compound **C42** contained two peaks due to the presence of two diastereomers (*L*-dopa ethyl ester *R*-indanone and *L*-dopa ethyl ester *S*-indanone), with an average proportion of 64:36 by order of elution. Solutions containing 0.77 mM of the mixture were tested for degradation. The pH/rate profiles for the two compounds are represented in Figure 5..

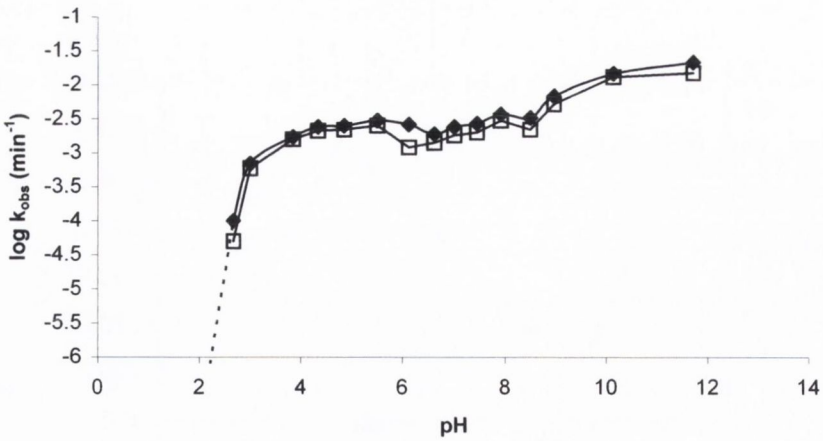


Figure 5.10: pH/rate profile of C42 (✦, first diastereomer, ⊟ second diastereomer)

The compounds are stable at pH=0.5 and no racemisation was observed over a two-day period.

The data for the average rate of the two diastereomers in the pH range 0.5-5.5 was fitted to equation **3.9** and afforded the profile depicted in Figure 5.11.

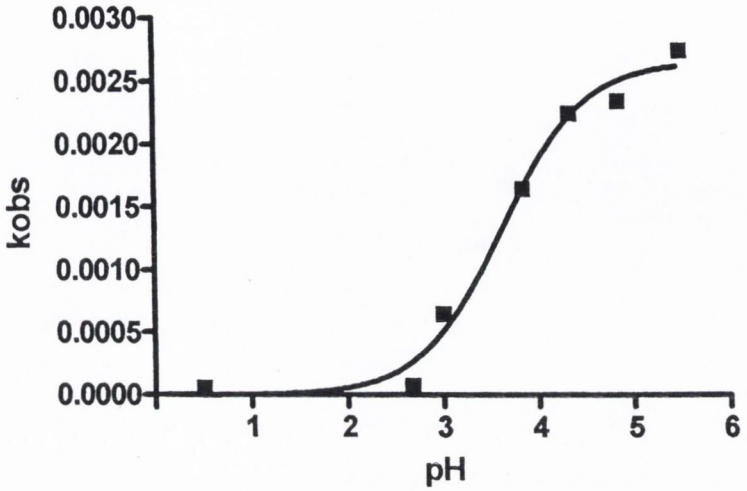


Figure 5.11: Fitting of equation 3.9 to the pH/rate profile of C42

The highest degradation rate afforded by this sigmoid is 0.0027 min^{-1}, which corresponds to a half-life of 260 minutes.

Maximum release of etilevodopa was found in the pH range 3.8-6.2 where the observed half-lives were in the order of 3.8 to 7.8 hours. While, above this pH, higher degradation rates were observed, lower levels of etilevodopa were detected. At pH from 7 to 8, there is evidence of etilevodopa degradation following release (Figure 5.12). This could be due to ester hydrolysis before and/or after release of the amine. However, if this was the case, the amino acid was not detected by the capillary electrophoresis conditions used, due to the simultaneous ionisation of the hydroxyl and the amine group.

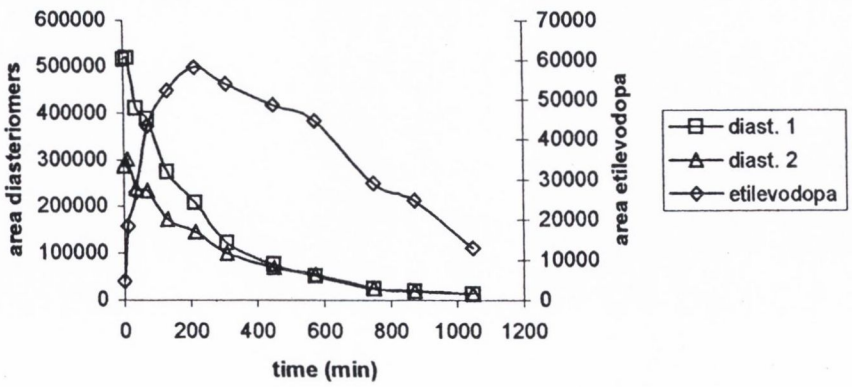


Figure 5.12: Degradation profile of the diastereomers of compound C42 at pH=8

Tests with compound **B16** revealed a pseudo-first-order rate of degradation at pH 7.4 of 0.0007 min^{-1}, which corresponds to a half-life of 16.5 hours.

Table 5.1 provides the parameters determined by fitting the data of the three prospective prodrugs, by non-linear regression.

Table 5.1: Parameters of the sigmoidal fitting of the pH/rate profiles of the derivatives of dopamine

compound	k_1 (min^{-1})	k_2 (min^{-1})	pK_a
C43	-0.00011±0.0012	0.0067±0.0030	6.59±0.92
C44	0.0012±0.0051	0. 12±0.016	6.99±0.22
C42	-7e-6±0.0003	0.0027±0.00034	3.74±0.35

5.4.2.2. Development and validation of a HPLC method for the quantification of dopamine

A HPLC method was developed and partially validated for the quantification of dopamine release from compounds **C43** and **C44**.

Initially, triethylamine containing eluents (previously used for other derivatives) were used. However, dopamine was not retained and therefore a system without triethylamine was preferred.

Eluents A and B consisted of 0.3% v/v phosphoric acid in water and methanol respectively. The gradient program is described in Table 5.2. The flow was 1.2 ml/min.

Table 5.2: Gradient HPLC program used for the prodrugs of dopamine

time	A (%)	B (%)
0	90	10
5	90	10
15	20	80
20	20	80
21	90	10
24	90	10
25	90	10

The temperature control of the autosampler and the column were kept off. Detection was made at 214 nm from 0 to 10 minutes and at 235 nm after that.

Calibration curves were drawn for dopamine at different pHs in the range of concentration of 0.0026 mM to 2.6 mM. The peak of dopamine was detected at approximately 3.3 minutes and was separated from the unretained peak with a separation factor of R_s=1.5. The capacity was 0.5.

Six determinations were performed at each pH and concentration. The average results and the corresponding confidence intervals (95%) are presented in Table 5.3.

These results were used for the determination of the calibration curves at each pH. The fitted equation and corresponding coefficient of determination are also presented in Table 5.3, together with the percentage residuals from the fitted curve at each concentration.

Table 5.3: Repeatability and linearity tests of dopamine at pH=0.5

Concentration (mM)	Retention time (min)	Peak area	RSD(%)	Residuals (%)
0.0026	3.347±0.002	21795	0.59	162
0.026	3.362±0.004	165783	0.27	23
0.066	3.393±0.009	386846	1.75	5.1
0.26	3.376±0.004	1622435	0.21	5.5
0.66	3.316±0.002	3870531	0.15	-1.7
1.3	3.296±0.009	7470838	0.2	-4.1
2.6	3.371±0.0012	15750104	0.28	1.0
Calibration curve	6.007e3C-22914	R^2=	0.9994	

The noise level of a blank sample at the retention time of dopamine was 0.012 mAU. A solution containing 0.066 μM of dopamine affords a peak with a height of 0.045 mAU, which is near the limit of detection (L_d, theoretically defined as the concentration that affords a peak three times the height of the noise level - 0.036 mAU). L_d should therefore be less than 0.066 μM or 0.012 mg/l of dopamine hydrochloride.

A 0.26 μM solution of dopamine affords a peak with a height of 0.19 mAU which is above the theoretical limit of quantification (L_q, defined as the concentration that affords a peak ten times the height of the noise level - 0.12 mAU).

The coefficient of determination obtained for the calibration curve confirms the linearity of the method. However, by using such a large range of concentrations for the calibration curve, the limit of quantification is compromised by the high residuals observed for low concentrations. This is due to the regression method used (minimum squares, not weighted), which minimises the absolute residuals and therefore affords larger relative residuals at lower concentrations. To overcome this, a different regression method should be used or a calibration curve should be obtained for a shorter range if the expected concentrations in the samples are low.

Table 5.4 and Table 5.5 present the results of repeatability and linearity tests at pH 3.5 and 7.4 respectively. At these pH another peak was observed at a shorter retention time, which

was due to components of the sample buffers. The separation factor of dopamine from this peak was R_s=1.1 in both cases.

Table 5.4: Repeatability and linearity tests of dopamine at pH=3.5

Concentration (mM)	Retention time (min)	Peak area	RSD (%)	Residuals (%)
0.0026	3.417±0	23634	2.26	309
0.026	3.448±0.002	183266	0.19	-45
0.26	3.376±0.004	1622435	0.21	-8.0
1.3	3.382±0.002	7161995	0.16	-5.9
2.6	3.337±0.002	15470711	0.13	-1.4
Calibration curve	5.878e3C-26590	R^2=	0.9986	

Table 5.5: Repeatability and linearity tests of dopamine at pH=7.4

Concentration (mM)	Retention time (min)	Peak area	RSD(%)	Residuals (%)
0.0026	3.368±0.006	15268	4.46	193
0.026	3.385±0.004	163805	0.25	24
0.26	3.364±0.005	1568361	0.94	3.9
1.3	3.318±0.003	7351507	0.40	-4.1
2.6	3.276±0.002	15490627	0.16	0.9
Calibration curve	5.913e3C-29611	R^2=	0.9994	

A test was performed also using pH=7.4 solutions quenched (1:1) with perchloric acid solution (5%). Results are presented in Table 5.6.

Table 5.6: Repeatability and linearity tests of dopamine at pH=7.4 in quenched solutions

Concentration (mM)	Retention time (min)	Peak area	RSD (%)	Residuals (%)
0.0026	3.515±0.008	6738	6.33	-169
0.026	3.602±0.008	70817	0.70	-9.9
0.26	3.577±0.005	778696	0.33	-4.3
1.3	3.541±0.002	3655658	0.25	3.2
2.6	3.511±0.001	7617571	0.35	-0.7
Calibration curve	2.913e3C-11952	R^2=	0.9996	

These solutions were left in the autosampler of the HPLC, which was heated to at 37°C. Upon repetition of the injections of the same solutions after two days, a reduction of 9% in the slope of the calibration curve at pH=7.4 was observed, suggesting degradation of

dopamine at this pH, with a half-life of 28.8 days. Differences in the slopes of the other curves were not considered important.

5.4.2.3. Degradation of 2-inden-1-one

No attempts were made to validate a calibration of 2-inden-1-one, as the compound was known to degrade.

Stability at 37°C was evaluated and afforded the pseudo-first-order decay profiles depicted in Figure 5.13.

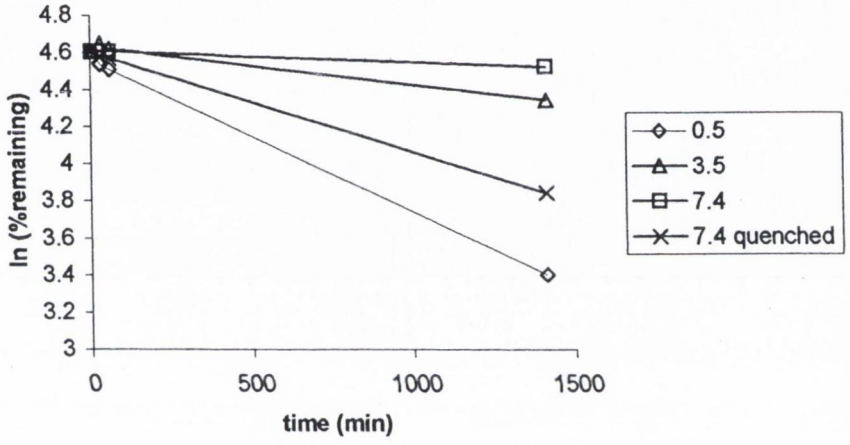


Figure 5.13: First order degradation plots of indenone at different pH

Indanone detected at RT=16.0 min, was found to be less stable in acidic than in neutral pH, with only one new peak forming at RT=12.0 min. The corresponding degradation rates and half-lives are presented in Table 5.7.

Table 5.7: Stability of indenone at different pH values

pH	Rate coefficient (min^{-1})	Half-life (h)
0.5	8.3e-4	13.9
3.5	2.0e-4	57.8
7.4	5.7e-5	202
7.4 quenched	5.4e-4	21.4

5.4.2.4. Determination of the pH rate profiles by HPLC

a) 3-Dopamine-indan-1-one (C43)

Dopamine-indanone was tested at different pHs over a period of four days. Solutions containing 1.18, 0.59 and 0.29 mM of the compound in different buffers were prepared from a stock containing 5 mg/1.5 ml in ACN/ buff. pH=3 (1:0.5).

The compound was stable at pH 0.5 and 3.1 (Figure 5.14). At these pHs, a 10% increase in the area of the compound was observed over this period. It was not established if this was due to a solubility effect or a steady change in the performance of the method.

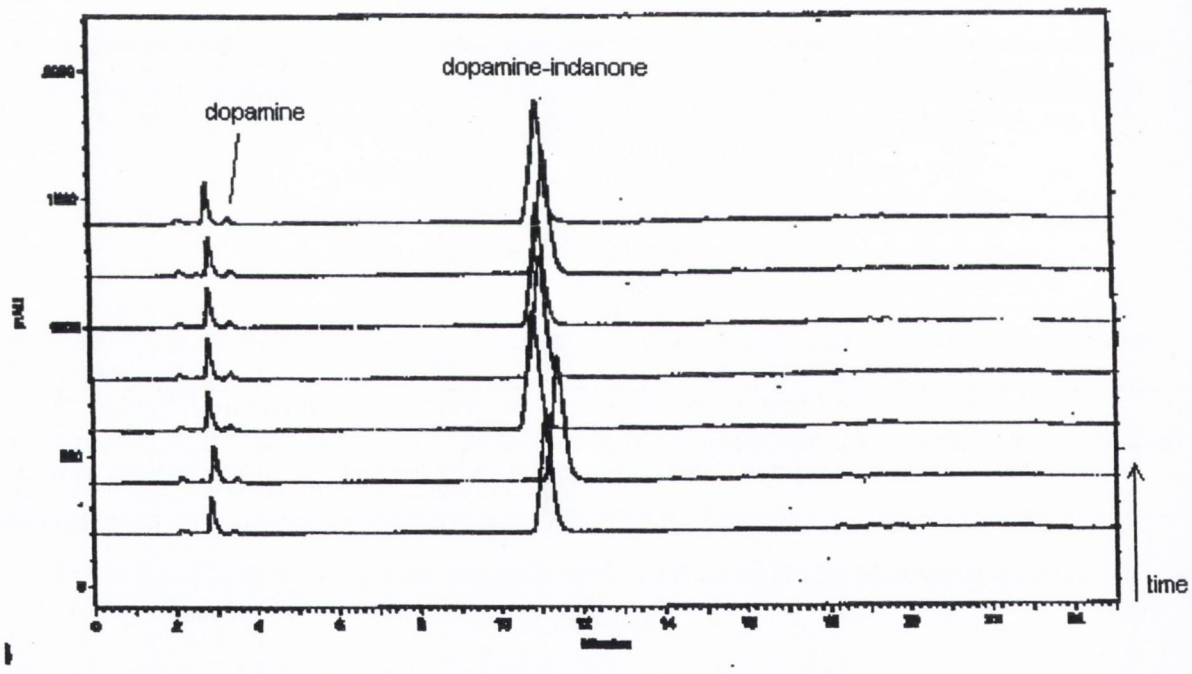


Figure 5.14: Chromatograms of a pH=3.1 solution of C43 over a 4 day period

At pH 4.5, the areas of the peaks of **C43** remained fairly constant over the test period but an increase in the areas of dopamine and indenone could be detected. This corresponded to release of 0.08 mM of dopamine or 6% degradation (half-life=48 days).

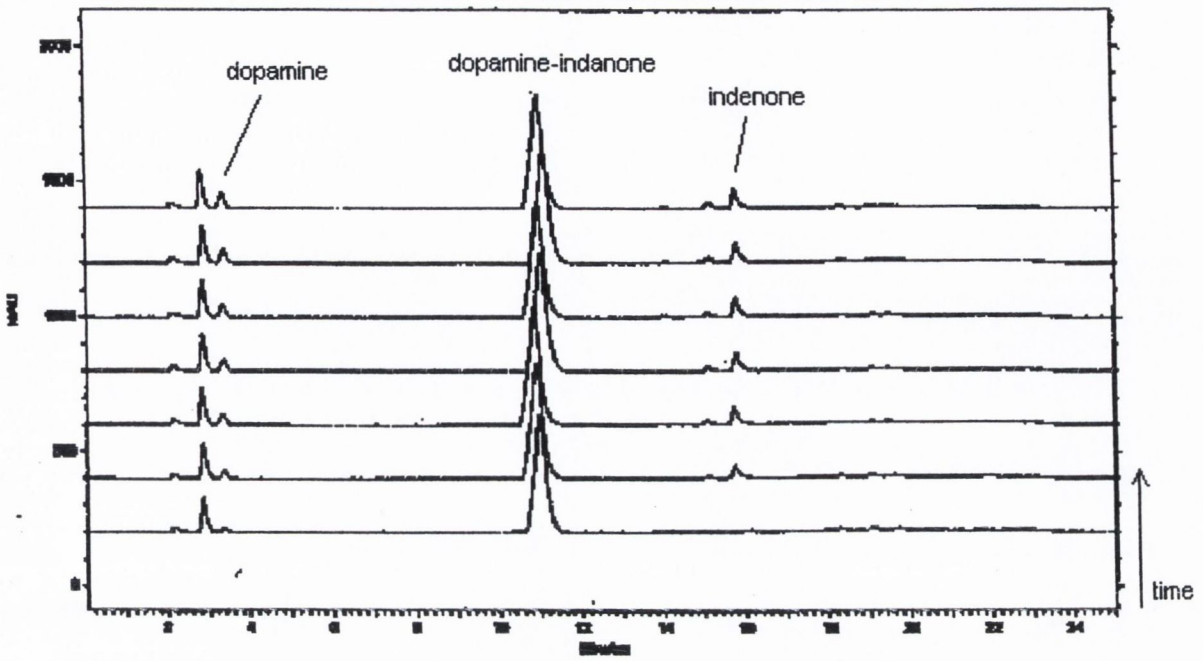


Figure 5.15: Chromatograms of a pH=4.5 solution of C43 over a 4 day period

At higher pH, the formation of new peaks was detected as illustrated in Figure 5.16 for pH 7.4. At this pH, and after total degradation of the prodrug, only 0.51 mM of dopamine was detected, which corresponds to a recovery of 44%.

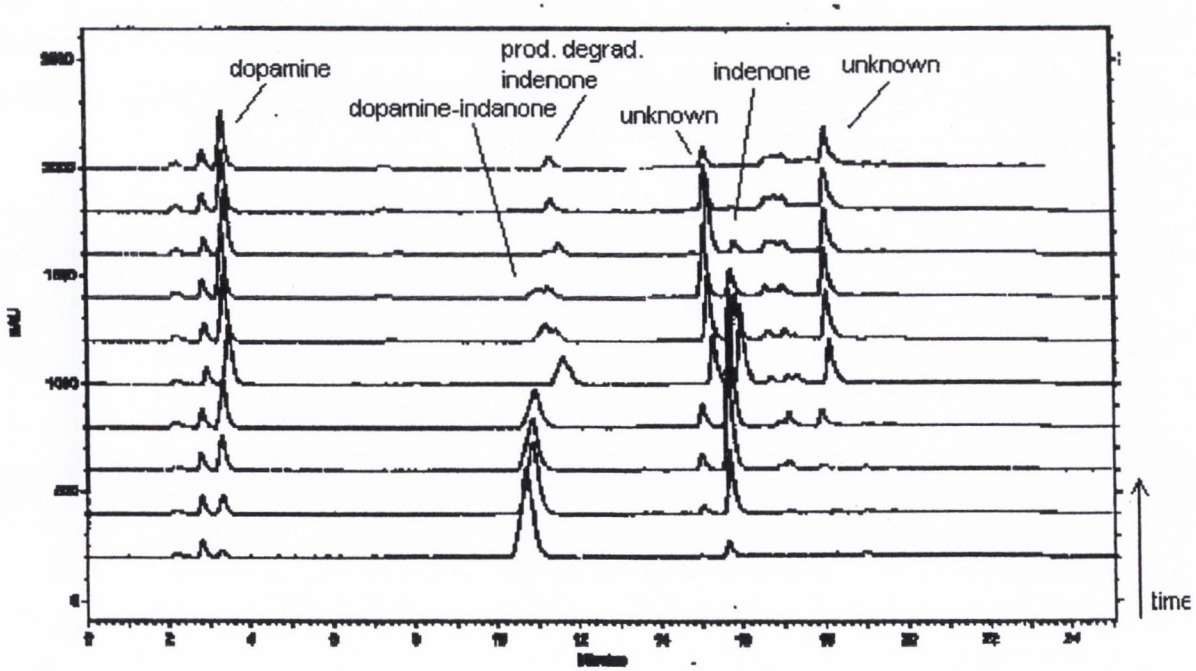


Figure 5.16: Chromatograms of a pH=7.4 solution of C43 over a 4 day period

As seen in the previous section, the low recovery of dopamine can, in part, be justified by its degradation after release. However, the appearance of new peaks suggests the degradation of **C43** by a different pathway or a consecutive reaction between the products of degradation of **C43**. Upon mixture of dopamine with indenone, the same unknown peaks were formed but no **C43** was detected, which rules out the intervention of this compound in the reactions that afford the unknown products.

Table 5.8: Results of the degradation studies of C43 at different concentrations and pH and corresponding release of dopamine

	Half-life	dopamine	Half-life	dopamine	Half-life	dopamine
pH	1.18 mM		0.59 mM		0.29 mM	
4.5	48 days	--				
6	29 h	59%	13 h	54%	14 h	47%
7	13 h	49%	4.9 h	46%	4.3 h	41%
7.4	13 h	44%	5.4 h	39%	5.4 h	38%
8	8.6 h	39%	3.2 h	41%	2.6 h	30%
9.5	2.2 h	21%	n.d.	n.d.	n.d.	n.d.
11	1.2 h	14%	n.d.	n.d.	n.d.	n.d.

Tests were performed using different initial concentrations of **C43**. Recovery of dopamine was calculated based on the maximum concentration observed and the percentage of **C43** that had degraded when that concentration was observed (usually around 80%). Figure 5.8 presents the results of these tests.

The rate of degradation of indenone at pH=7.4, after the maximum concentration was observed, was more that 10 times faster than the rate of degradation observed in tests in solutions containing indenone exclusively ($t_{1/2}$=15.7h). The degradation still appeared to follow first order kinetics but this may be masked by simultaneous production and degradation of the compound.

Tests were also performed at different ionic strengths and pH=7.4 in solutions containing 0.59 mM of **C43**. Results are presented in Table 5.9.

On comparison of the data in Table 5.8 and in Table 5.9, a discrepancy is observed for the results at I=0.154. No explanation was determined for this. However, since indenone is known to be photosensitive, one hypothesis is that the light conditions in the lab may have affected the rates of degradation. The ionic strength tests were started between midday and two o'clock, while the concentration tests started after four in the afternoon (on a

different day). However there is no registry available of the light conditions during those days.

Table 5.9: Results of the degradation studies of C43 at pH=7.4 and at different ionic strengths and corresponding release of dopamine

I	Half-life	dopamine
0.5	6.7 h	35%
0.25	4.4 h	31%
0.154	4.4 h	27%
0.05	4.0 h	27%
0.0154	2.6 h	21%

b) Dopamine-benzyl acetophenone (C44)

A stock solution of 4.2 mg/1.5 ml of **C44** in ACN/ buff. pH=3 (1:0.5) was diluted in buffers of different pH to obtain concentrations of 0.35 mM of **C44**. The compound was stable at pH=0.5, but degraded at pH=3.1 although slowly.

Only chalcone and dopamine were detected upon degradation of compound **C44** at all pHs. Figure 5.17 presents the chromatograms obtained at pH=4.4.

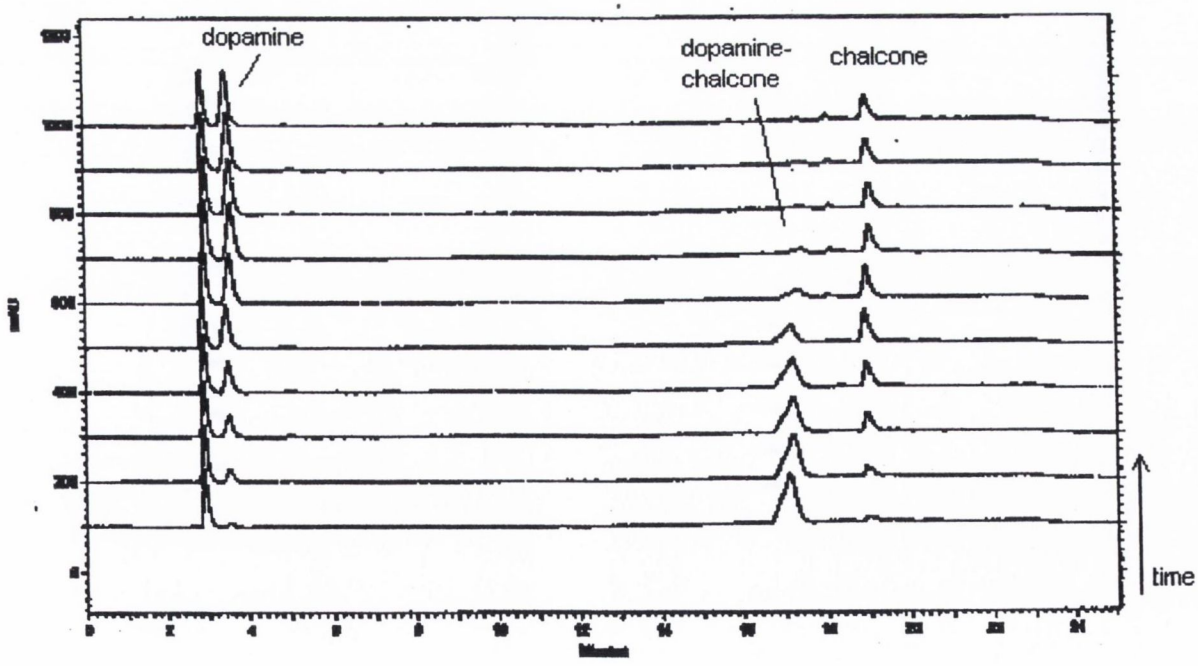


Figure 5.17: Chromatograms of a pH=4.4 solution of C44 over a 3 day period

Elimination from **C44** was much faster than from **C43** at all pHs and the percentage of recovered dopamine was higher which can be seen in Table 5.10.

Table 5.10: Results of the degradation studies of C44 at different pH and corresponding release of dopamine

pH	Half-life	dopamine
3.1	4.8 days	86%
4.4	8.7 h	66%
6	23 min	58%
7	12 min	57%
7.4	14 min	59%
7.9	10 min	62%
9.5	19 min	50%
11	14 min	23%

5.4.2.5. Elimination of dopamine in plasma

Compounds **C43** and **C44** were also tested for elimination of dopamine in plasma solutions. Stock solutions of 5.0 mg /1.5 ml and 4.2 mg/1.5 ml in ACN: buffer pH=3.1 (1 ml: 0.5 ml) respectively were used. Aliquots of 150 μl and 130 μl of **C43** and **C44** respectively, were diluted in 3 ml of plasma and buffer pH=7.4 at 37°C to afford concentrations of 0.56 mM and 0.29 mM respectively (three samples each). Aliquots were taken immediately (0.2 ml), quenched with 0.3 ml of a solution of 5% perchloric acid in water, homogenised and centrifuged at 10000 r.p.m. for 3 minutes (final concentrations of **C43** and **C44** are 0.22 mM and 0.12 mM respectively). The plasma and buffer samples were incubated at 37°C and new aliquots were taken at time intervals and treated as previously described. These deproteinised samples were analysed by HPLC using the method described in 5.4.2.2.

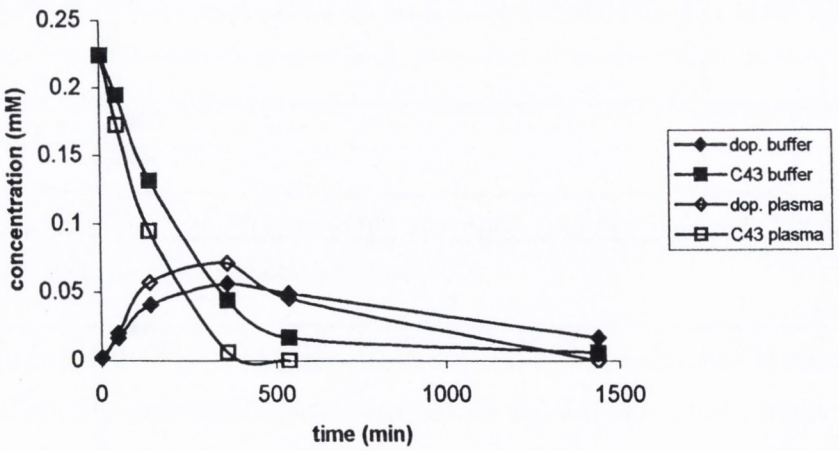


Figure 5.18: Degradation profiles of C43 in buffer and plasma

The profiles of degradation of **C43** in plasma and buffer are represented in Figure 5.18. Concentrations of dopamine were calculated based on a calibration curve prepared in the same day. Faster degradation of dopamine, in quenched solutions in comparison with non quenched solutions (Figure 5.16) may be related to side reactions with the other degradation products.

As seen before in buffer samples, compound **C44** degraded faster than **C43** and afforded higher percentages of dopamine as depicted in Figure 5.19.

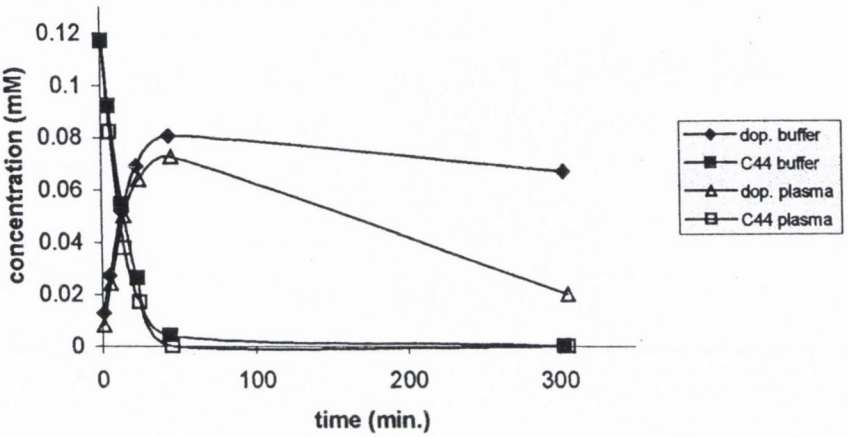


Figure 5.19: Degradation profiles of C44 in buffer and plasma

Percentage results for recoveries of dopamine from plasma and pH 7.4 buffer solutions are presented in Table 5.11.

Table 5.11: Half-lives of C43 and C44 and corresponding recoveries of dopamine (average of three determinations)

	pH	Half-life	dopamine
C43	buffer	2.38±0.27 h	25.0±2.8%
	plasma	1.82±0.25 h	31.7±0.24%
C44	buffer	8.8± 1.0 min	68.8±2.1%
	plasma	8.3±0.6 min	62.1±3.6%

The half-lives observed in these off-line rate determinations were shorter than the rates observed during the pH/rate profile determinations in non-quenched samples. This is probably due to difficulties in the accurate determination of temperature inside sample vials during on-line tests, as opposed to a more accurate control of temperature in off-line treated samples.

Recoveries of dopamine were generally larger for **C44** and were the same order of magnitude in buffer and plasma samples. However, in the case of **C43**, half-life in plasma was shorter than in buffer.

Degradation of dopamine following elimination can contribute to the low recoveries observed, particularly in the case of compound **C43** where elimination is slower.

Indenone was detected upon degradation of **C43** in buffer but not in plasma. The same was observed with chalcone for compound **C44**. This may reflect the Michael acceptor properties of enones with fast addition of proteins. Binding to protein and alkylation of these compounds with cellular thiol such as glutatione is a commonly known phenomenon, which has been cited several times in the literature[321,234,323,324].

5.4.3. Tests in vivo

5.4.3.1. Absorption in the everted rat gut

Compound **C43** was tested for absorption in the everted rat gut (three animals). 50 mg were dissolved in 1 ml of DMSO and diluted in 66 mM phosphate buffer, pH 7.4 (100 ml).

This solution was administered to each rat through a cannula at 0.2 ml/min for two hours. The perfusate was collected at ten-minute intervals over the test period. Each sample collected was weighed and quenched with two ml of a solution containing perchloric acid (4%) and EDTA (1 g/l). **C43** was quantified by CE, in each of the samples, as a percentage of the concentration contained in the administered solution. The concentration of the perfusate at the endpoint of the experiment was used as a reference to account for any degradation occurring during the experiment.

Figure 5.20 represents the concentration of **C43** in the perfusate samples collected after passage in the intestine as a fraction of the concentration of the same solution not subjected to the experiment.

Membrane disruption was not tested in this case. For this reason it is not possible to conclude if there was concomitant water absorption (as with the 1-aminoindane derivatives - Section 3.11) and if the observed 10 and 30% absorption of compound in solution were real or in fact higher.

The permeability coefficient calculated using equation 3.27 and based on the last three points of each curve is 2.3e-5 cm/s and has a RSD of 8%.

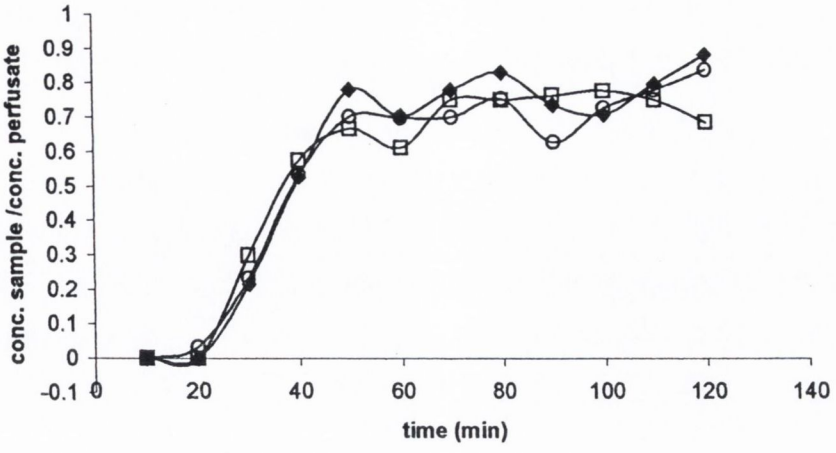


Figure 5.20: Plots of the remaining percentage of C43 after perfusion (three rats)

5.4.3.2. Intravenous administration

a) Blood tests

A solution of **C43** of 26.8 mg/ml was prepared in saline/ethanol/DMSO/propylene glycol (1:0.5:0.1:0.4) for administration in the form of a bolus injection.

Injections of 0.1 ml were administered to two animals and blood samples were collected after 15 and 30 minutes respectively. To another animal, a bolus injection of 0.2 ml was administered and a blood sample collected after 15 minutes.

Plasma was separated from the blood samples and 1 ml aliquots were quenched with 1.5 ml of the quenching solution containing perchloric acid and EDTA, homogenised and centrifuged for 3 minutes at 10000 r.p.m.. The samples obtained in this manner were analysed by CE. **C43** was not detected but a small peak that could be due to dopamine was observed although not quantifiable.

This was thought to be due to binding of **C43** to blood proteins and consequent elimination from the plasma samples sequentially tested. No previous tests had been performed where blood samples were spiked directly with the prodrug.

For this reason, a new test was developed that consisted of spiking a human blood sample with 0.531 mg/ml of **C43** and then treating it in two different ways: 1) separation of plasma and posterior quenching and analysis as described previously and 2) direct quenching of blood followed by centrifuging and analysis. In order to obtain clear extracts, 0.5 ml blood

samples were quenched with 1.5 ml of quench solution. Plasma samples (0.5 ml) were quenched with 0.75 ml of quench solution.

C43 was found to degrade much faster in blood-spiked samples than was observed in spiked plasma samples (cf. Table 5.11). A peak for dopamine was detected but not quantified. The rates of degradation were 9 min in blood samples quenched directly and 17 min in blood samples from which plasma was separated before quenching. The degradation profiles based on the remaining percentage of the original peak in these two tests and a control in buffer solution is presented in Figure 5.21.

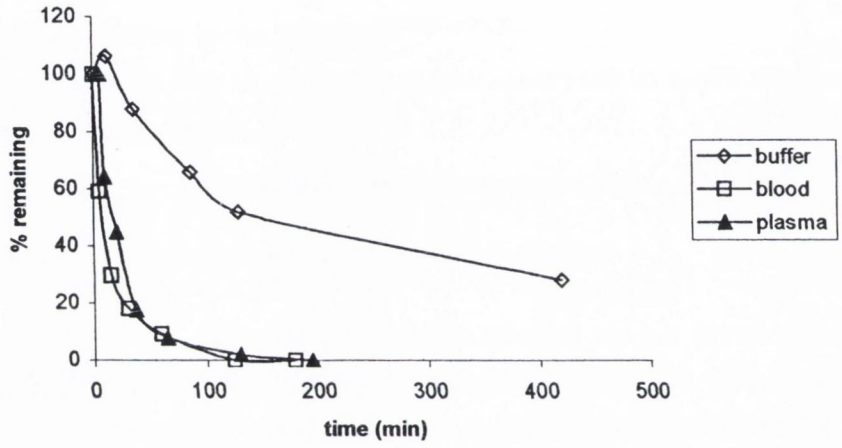


Figure 5.21: Profile of elimination of C43 from buffer and blood samples (quenching before or after plasma separation)

The fact that **C43** degrades faster in spiked blood samples than in spiked plasma samples may explain why the compound was not detected in rat blood. Moreover degradation in rat blood may follow a different profile than degradation in human blood.

One possibility is that metabolism of the catechol group is responsible for the rapid decay and alternatively that the compound binds to blood proteins and consequently its availability is reduced.

b) Brain tests

The brain of each animal was collected after being perfused with saline solution to eliminate any blood contaminating the tissue. It was then macerated using an equivalent mass of quenching solution and the supernatant was separated by centrifugation. The samples were analysed by CE but neither dopamine nor **C43** were detected. However the compounds were detectable in tests in spiked brain samples.

5.4.4. Determination of the pKa

The pK_as of the test compounds were determined in the same manner as described in the previous chapters. Results are presented in Table 5.12.

A second ionisation, which is due to one of the hydroxyl groups, was detected as a reversal in the direction of mobility as illustrated for compound **C42** (Figure 5.22) but was not quantified due to the imperfect shape of the sigmoidal curves and difficulties in evaluating the final mobility at higher pH.

Table 5.12: pK_as of the derivatives of dopamine as determined by capillary electrophoresis

amine	pK_a amine (lit.)	pK_a amine (exp.)	compound	pK_a (exp.)
dopamine	8.8, 10.6[170]	7.59±0.14, n.d.	**C43**	6.81±0.10, 8.53±0.26
			C44	6.97±0.10, 8.69±0.20
etilevodopa (**B16**)	7.12[beilstein]	6.74±0.09, 8.86±0.20	**C42**	4.18±0.11, 8.11±0.12

The first ionisation constants obtained in this manner were in good agreement with the pK_as obtained from non-linear fitting of the pH/rate constant profiles (cf. Table 5.1).

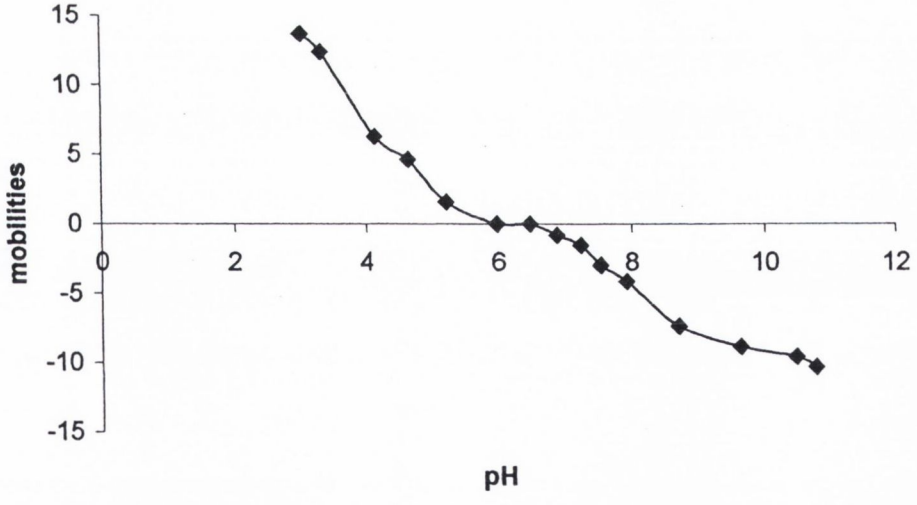


Figure 5.22: Ionisation profile of compound C42

In the case of compounds **C43** and **C44**, the two ionisation constants are closer in the pH range (Figure 5.23). If the general form of the Boltzmann equation (equation **A.20**, Appendix 1) was used for the determination of the pH, the accuracy of the determination of the first ionisation constant could have been compromised. This is because the

simultaneous ionisation of the amino and the hydroxyl group could lead to the faster decay to zero mobility than would be observed in the absence of the second ionisation.

Some authors have applied corrections to account for multiple ionisation. However, this was not considered necessary when using the particular case of Boltzmann (equation **3.14**). In this instance, the slope of the sigmoid is fixed and the correct fit should be obtained even if good data is available only for the first portion of the sigmoid as long as the final mobility is known. In order to determine the first pK_a of these compounds, zero mobilities were assumed for pH values above eight, when fitting equation **3.14**.

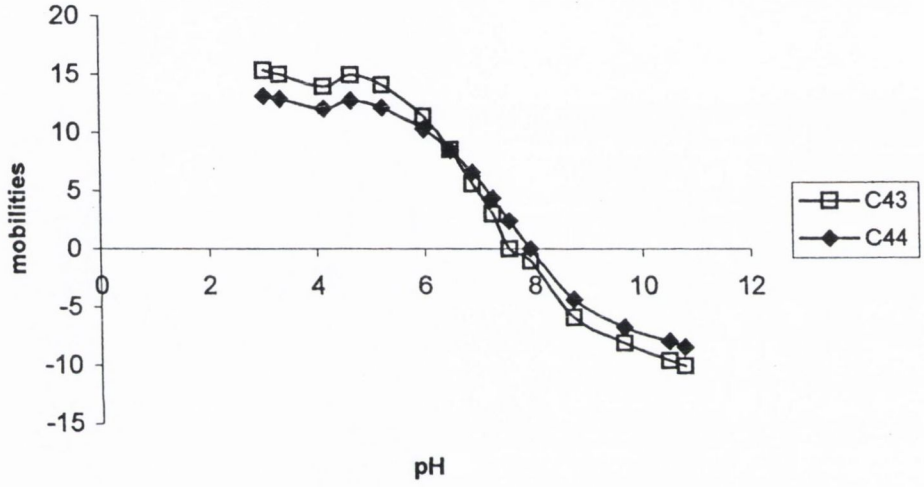


Figure 5.23: Ionisation profile of compounds C43 and C44

5.4.5. Estimation of log P

As was evident for other compounds described in previous chapters, derivatisation of the drugs with indanone or benzyl acetophenone promoieties affords substantial increase in the lipophilicities of the basic form of the compounds.

Table 5.13: Estimations of log P for the test compounds and free amines

amine	log P (lit.)	log P (est.)	compound	log P (est.)
dopamine	-2.48*	0.70	**C43**	1.99
			C44	4.02
L-dopa ethyl ester		0.60	**C42**	2.15

* from Chemdraw database

5.5. Conclusions

Prodrugs containing the indanone promoiety degraded slowly (with half-lives of approximately two and four hours for compounds **C43** and **C42** respectively) at neutral to basic pHs and may be suitable for sustained delivery of dopamine. The chalcone derivative (**C44**) degraded much faster ($t_{1/2}$=8 minutes approximately) but its acid stability still makes it suitable to withstand the low pH of the stomach.

Low recovery of the free amines may be due to further degradation of dopamine following release or hydrolysis in the case of etilevodopa. Higher recoveries of dopamine observed for **C44** in comparison with **C43** are probably due to faster rates of elimination that overcomes the degradation rate of dopamine. Further tests need be carried out on derivatives protected at the catechol group, for example by acetylation as in the case of docarpamine.

Although there is no clear synergistic effect on degradation in plasma samples, **C43** is eliminated much faster from blood samples. This may be due to a different path of degradation or to binding to blood proteins. In the former case, if binding is reversible, this may provide further protection against degradation and sustained release of the prodrug to the blood stream. Further investigation is necessary to confirm this.

C43 was evaluated for absorption in the rat gut and only about 20% of the compound were found to be absorbed. This may compromise the bioavailability of the drug upon oral administration. On the other hand, high lipophilicity and low solubility may render the compound unsuitable for intravenous administration.

The compound was not detected in rat brain following intravenous administration but this may be a consequence of the fast withdrawal from solution in blood. Ultimately, only behavioural tests will provide the answer to the question of whether the drug passes the BBB and is suitable for treatment of Parkinson's disease.

CHAPTER 6. CONCLUSION REMARKS AND FUTURE WORK

We have evaluated a novel prodrug system for amines. The system consists in aromatic β-aminoketones, which are stable in acidic pHs but are chemically activated in basic medium releasing the amines by a retro-Michael reaction. On the other hand the compounds seem to be stable towards P450 metabolism.

The retro-Michael reaction is well known but it has not been applied previously in this context, probably because the rates of reaction are prohibitively slow for most derivatives.

We have found a group of promoieties that release the amine particularly fast and this may be due to the high level of conjugation of the final α,β-unsaturated ketone. These include derivatives where the amine is in a benzylic position and therefore, upon elimination, the double bond is formed in conjugation with the benzene ring and the carbonyl group.

The rates of the elimination reaction were evaluated by capillary electrophoresis and/or HPLC. Although HPLC afforded more robust methods, CE is useful for quick profiling and for evaluation of chiral compounds.

Derivatives of 1-indanone and of benzyl acetophenone proved the most promising prodrugs and will be discussed further ahead. Amino benzyl acetones may degrade at rates similar to amino benzyl acetophenones but their inherent instability may pose several synthetic problems.

Mannich bases of acetophenone do not share the same conjugation features of the previously mentioned compounds and therefore they are thought to display slower

release rates. They could, nevertheless, be useful to produce prodrugs of bulky secondary amines (such as atenolol) that in some cases may be eliminated too fast from higher conjugated systems like indanone. However, vinyl ketones which would be obtained upon elimination from this system, are known mutagenic compounds and therefore this line of investigation may not be worthwhile to pursuit.

Ester derivatives of propionic acid were also studied as promoieties but no amine was found to be eliminated from this system. This is thought to be due to a faster rate of ester hydrolysis and provides some evidence that amine elimination from non-conjugated systems may be too slow. It would be interesting to study the behaviour of 3-amino-3-phenyl propionates as this may afford an aromatic β-aminoester alternative to β-aminoketone systems.

As previously stated, β-aminoindanones and 3-amino-1,3-diphenyl-propan-1-ones (derivatives of benzyl acetophenones) constitute the most promising compounds. The former compounds generally eliminate at slower rates than the latter and may be useful as alternatives to slow release formulations. The latter usually display half-lives of only a few minutes at basic pHs but, since they are still considerably stable at low pHs they may be adequate to afford protection to the amino group at low pHs.

Indanone or benzyl acetophenone derivatives have ionisation constants that are generally three units below the ionisation constants of the free amines. The prodrugs are also considerably more lipophilic than the original compounds. These two characteristics should favour the passage through biological membranes and we have observed that, at least in the case of the indanone prodrug of 1-aminoindane, absorption is improved by prodrug preparation. However, high lipophilicity may pose, in some cases, important solubility problems.

Some authors have observed that substitution in the benzene ring affects the rates of elimination. This may be a new line of work to pursuit in order find ways of controlling the release rates.

Further studies are needed on the activity and toxicity of the progroup. Chalcone has been widely studied and it has been referred to have several biological effects but no general conclusion on its toxicity has been found. On the other hand, no biological studies were found on 2-inden-1-one. During the course of this work we have seen that indenone degrades in buffer solutions but the degradation compounds were not identified. Chalcone appeared more stable.

Unsaturated ketones are known to be highly reactive compounds and, in some cases such as previously mentioned vinyl ketones, they were even found to be toxic. However, their high reactivity may also work in favour of the rapid formation of easily excretable metabolites.

The indenone prodrugs of dopamine and L-dopa described in Chapter 5 may be suitable for the sustained delivery of dopamine either for the treatment of Parkinson's or cardiovascular diseases. More tests have to be performed in order to evaluate how this type of derivatisation affects passage through the BBB. Nevertheless, transport across this membrane is not indispensable in the case of the L-dopa derivative since the amino-acid can still penetrate into the brain after being released from the promoiety.

The rates of elimination from the benzyl acetophenone derivative of dopamine are too high for this purpose but may be useful to afford protection of the amino group before absorption and for increasing the bioavailability of the drug upon oral administration. Comparative tests with dopamine have to be performed to confirm this possibility.

Behavioural tests are necessary to further evaluate the adequacy of the indenone derivatives for the treatment of Parkinson's disease.

These sustained release prodrugs may need further derivatisation in order to protect the catechol group against metabolism during the high residence times in the organism.

CHAPTER 7. EXPERIMENTAL

7.1. Chemistry

7.1.1. Materials

Solvents used for synthesis were distilled in-house and reagents were of general analytical grade. These included: D,L-alanine methyl ester hydrochloride, L-phenylalanine benzyl ester p-toluenesulfonate salt, 3-hydroxytyramine hydrochloride, D,L-tryptophan methyl ester hydrochloride all from Sigma; 1-indanone 99%, 2-methyl-1-indanone 99%, 1-aminoindane, acetophenone 99%, 4-phenyl-2-butanon 98%, Methyl 3-bromopropionate 97%, L-3,4-dihydroxyphenylalanine 99%, 2-phenylethylamine 99%, benzoyl peroxide (70% remainder water), and 1,1´azobis(cyclohexane carbonitrile) 98% from Aldrich; 2-aminoindane hydrochloride >98%, benzalacetone >98%, S(+)-1-aminoindane, R(-)-1-aminoindane, benzalacetophenone >98%, 4-(4-hydroxyphenyl)-2-butanone >97% from Fluka; Atenolol for medicines preparation from Societá Italiana Medicinali Scandicci; loratadine and desloratadine micronised USP from Schering-Plough (Avondale) company.

Buffers for kinetic and everted rat gut tests were prepared from citric acid monohydrate 99% ACS from Aldrich, tripotassium orthophosphate from BDH, sodium tetraborate from M&B, di-natrium hydrogenphosphate-2 hydrate, natrium dihydrogen phosphate, hydrochloric acid and sodium chloride from Riedel de-Haën.

Running buffers for capillary electrophoresis were prepared from lauryl sulfate sodium salt 99% (for EC), β-cyclodextrin (min 98%), heptakis(2,3,6-tri-o-methyl)-β-cyclodextrin (min 90%), heptakis(2,6-di-o-methyl)-β-cyclodextrin (min 40%) from Sigma;

hydroxypropyl-β-cyclodextrin (M.S.=0.8), sulfated β-cyclodextrin (sodium salt), taurodeoxycholic acid, sodium salt hydrate 95%, tetrabutylammonium dihydrogen phosphate 97% from Aldrich and Urea AnalaR 99.5% from BDH.

HPLC grade solvents from Riedel-de Haën and distilled and deionised water (Milli-Q) were used for analytical work.

7.1.2. Characterisation of compounds

Final compounds, where new, were fully by infrared spectroscopy, nuclear magnetic resonance (^1H and ^{13}C), mass spectroscopy and melting point. In some cases, cosy spectrums were also obtained to assist the assignment of proton or carbon shifts. Intermediates and non-target products were not always fully characterised.

Infrared spectrums (IR) were obtained using a Perkin Elmer Paragon 1000 FT infrared spectrometer. Samples were analysed as neat films from dichloromethane on NaCl plates (oils) or in KBr tablets (solids). Wave numbers (IR ν_{max}) are presented for characteristic functional groups and are expressed in cm^{-1}.

^1H, ^{13}C, cosy and tocsy NMR spectra were recorded at 20°C on a Brucker DPX 400 spectrophotometer (400.13 MHz ^1H, 100.61 MHz ^{13}C) at the Department of Chemistry, Trinity College Dublin. Samples were dissolved in deuterated chloroform ($CDCl_3$) or deuterated dimethyl sulfoxide ($(CD_3)_2SO$). Chemical shifts are in ppm. Coupling constants are in Hertz. ^1H shifts were assigned relative to the tetramethylsilane (TMS) peak at 0.00 ppm and ^{13}C shifts were assigned relative to the central carbon of the $CDCl_3$ triplet at 77.0 ppm or relative to the middle $(CD_3)_2SO$ septet at 39.7 ppm.

^1H NMR assignments are reported in the following form: shift values (number of protons, multiplicity and shape, coupling constant (when applicable), proton assignment). ^{13}C NMR assignments are reported in the following form: shift values (number of carbons (if more than one), carbon assignment). Aromatic protons and carbons are termed Ar<u>H</u> and Ar<u>C</u>H respectively.

In the cases were diastereomers were obtained and not separated, an asterisk is used as a superscript after the shift to indicate that it corresponds to the dominant diastereomer or to the mixture. The shift values for the minor diastereomer are not accompanied by an asterisk. When both diastereomers seem to be in the same proportion, the asterisk is used only to indicate that the particular shift corresponds to a different diastereomer than the shift previously assigned to the same proton(s).

High-resolution mass spectra (HRMS) were acquired on a time-of-flight (TOF) Micromass spectrometer at the Department of Chemistry, Trinity College Dublin. Leucine enkephalin acetate (FW=555.6) from Sigma was used as an internal lock mass reference. Acetonitrile was used as carrier.

For some volatile compounds, mass spectra were obtained on a Varian Chrompack CP-3800 GC equipped with a MS-Saturn 2000 ion trap detector. A DB-35 MS fused silica column with an internal diameter of 0.25 mm and 30 m long was used. The injector temperature was 260°C. The column was at 100°C for 6 minutes, after which the temperature was increased to 300°C at 15°C/min and kept for another 10 minutes. The carrier gas was helium. In these cases the masses of the main peaks corresponding to the most probable fragments are presented.

Uncorrected melting points (MP) were obtained using an Electrothermal apparatus and are expressed in Centigrade.

UV spectroscopy was carried out on a Cary 3E UV-Vis spectrophotometer.

X-ray crystallography was carried out in the Department of Protein Crystalography at the Instituto de Tecnologia Química e Biológica, Universidade Nova de Lisboa, Portugal.

7.1.3. Synthesis

Flash column chromatography was carried out on Merck kieselgel 60 (particle size 0.040-0.063 mm). Thin layer chromatography (TLC) was carried out on silica gel Merck F-254 plates.

General Procedure 1: <u>preparation of 3-Bromo-indan-1-one and 3-Bromo-2-methyl-indan-1-one</u>

The brominated compounds were prepared by refluxing the non-brominated ketones (1-indanone or 2-methyl-1-indanone) with N-bromosuccinimide (NBS) and a catalytic amount of dibenzoyl peroxide (approx. 0.1 g), in carbon tetrachloride (100 ml).

Equimolar concentrations of the ketones and NBS were used. After 45 minutes the reaction mixtures were cooled, filtered and evaporated. The residues obtained were then cleaned up by flash column chromatography on silica gel, using a gradient elution from 100% hexane to 10% ethyl acetate in hexane. The fractions containing the products afforded yellow oils by evaporation that were than kept in the freezer until further use.

3-Bromo-indan-1-one (B01) was prepared from 1-indanone (5.28 g, 40 mmol) and NBS (7 g, 40 mmol) as described in General Procedure 1. A yellow oil was obtained with a yield of 53% (4.45 g, 21 mmol). IR ν_{max} (NaCl plate) 1719 (C=O) cm^{-1}. δ_H ($CDCl_3$), 3.05 (1H, dd, J_{gem}=19.8, J_{vic}=2.5, C$\underline{H}_2$), 3.36 (1H, dd, J_{gem}=19.8, J_{vic}=7.5, C$\underline{H}_2$), 5.61 (1H, dd, J=2.5, 7.5, C$\underline{H}$Br), 7.49 (1H, m, C$\underline{H}$CHCHCC=O), 7.71 (2H, m, C$\underline{H}$CHC$\underline{H}$CHCC=O), 7.75 (1H, d, J=8.0, C$\underline{H}$CC=O). δ_C (CDCl3) 40.6 ($\underline{C}$HBr), 48.0 ($\underline{C}H_2$), 123.3 ($\underline{C}$HCC=O), 127.2 ($\underline{C}$HCHCHCHCC=O), 129.6 ($\underline{C}$HCHCHCC=O), 135.9 ($\underline{C}$HCHCC=O), 135.9 ($\underline{C}$CHBr) 154.2 ($\underline{C}$C=O), 201.5 ($\underline{C}$=O).

2-Bromo-indan-1-one (B02) was obtained as a co-product during preparation of 3-Bromo-indan-1-one. Yellow oil 24% (2.02 g, 9.5 mmol). IR ν_{max} (NaCl plate) 1723 (C=O) cm^{-1}. δ_H ($CDCl_3$), 3.44 (1H, dd, J_{gem}=18.0, J_{vic}=3.0, C$\underline{H}_2$), 3.86 (1H, dd, J_{gem}=18.0, J_{vic}=7.5, C$\underline{H}_2$), 4.67 (1H, dd, J=3.5, 7.5, C$\underline{H}CH_2$), 7.43-7.51 (2H, m, C$\underline{H}$C$\underline{H}$CHCHCC=O), 7.69 (1H, t, J=7.0, C$\underline{H}$CHCC=O), 7.86 (1H, d, J=7.5, C$\underline{H}$CC=O). δ_C ($CDCl_3$) 37.9 ($\underline{C}H_2$), 44.0 ($\underline{C}HCH_2$), 125.0 ($\underline{C}$HCC=O), 126.4 ($\underline{C}$HCHCHCHCC=O), 128.2 ($\underline{C}$HCHCHCC=O), 133.5 ($\underline{C}$HCHCC=O), 135.9 ($\underline{C}$CHCHCHCHCC=O), 151.1 ($\underline{C}$C=O), 199.6 ($\underline{C}$=O). *m/z* 102 (M^+-HBr-O=C••, 21%), 131 (M+-Br.•, 65%), 211 (MH^+ (^{79}Br), 100%), 213 (MH^+ (^{81}Br), 72%).

3-Bromo-2-methyl-indan-1-one (B06) was prepared from 2-methyl-1-indanone (1.46 g, 10 mmol) (General Procedure 1) and the product was obtained with a yield of 64% (1.43 g, 21 mmol). IR ν_{max} (NaCl plate) 1720 (C=O) cm^{-1}. δ_H ($CDCl_3$), 1.42 (3H, d, J=7.52, C$\underline{H}_3$) 3.01 (1H, m, C$\underline{H}CH_3$), 5.13 (1H, d, J=4, C$\underline{H}$Br), 7.50 (1H, m, C$\underline{H}$CHCHCC=O), 7.72 (2H, m, C$\underline{H}$CHC$\underline{H}$CHCC=O), 7.75 (1H, d, J=7.6, C$\underline{H}$CC=O). δ_C ($CDCl_3$) 13.3 ($\underline{C}H_3$) 49.3 ($\underline{C}$HBr), 54.6 ($\underline{C}HCH_3$), 123.4 ($\underline{C}$HCC=O), 127.3 ($\underline{C}$HCHCHCHCC=O), 129.6 ($\underline{C}$HCHCC=O), 135.5 ($\underline{C}$HCC=O), 143.2 ($\underline{C}$CHCHCHCHCC=O), 152.1 ($\underline{C}$C=O), 203.0 ($\underline{C}$=O).

2-Inden-1-one (B03) 3-bromo-indan-1-one (0.42 g, 2 mmol) was dissolved in dry ether (50 ml) and 1.1 equivalent of triethylamine (0.22 g, 2.2 mmol) was added. The solution was stirred for two hours at room temperature. The precipitate of triethylamine bromide was removed by filtration and the solvent was evaporated. The residue was purified by flash chromatography, using hexane/ethyl acetate (9:1) as mobile phase and a yellow oil was afforded with 73% yield (0.19 g, 1.5 mmol). δ_H ($CDCl_3$) and δ_C ($CDCl_3$) is in agreement with the literature[325]. IR ν_{max} (NaCl plate) 1714 (C=O) cm^{-1}. δ_H ($CDCl_3$) 5.87 (1H, d, J=5.5, CH=C$\underline{H}$C=O), 7.05 (1H, d, J=7.0, C$\underline{H}$=CHC=O), 7.22 (1H, t, J=7.3, C$\underline{H}$CHCHCC=O), 7.33 (1H, t, J=7.5, C$\underline{H}$CHCC=O), 7.41 (1H, d, J=7.0, C$\underline{H}$CHCHCHCC=O), 7.56 (1H, d, J=6.0, C$\underline{H}$CC=O). δ_C ($CDCl_3$) 122.1 ($\underline{C}$HC=O), 122.4

(CHCHCHCHCC=O), 127.0 (CHCHCC=O), 129.0 (CHCC=O), 130.1 (CCHCHCHCHCC=O), 133.5 (CHCHCHCC=O), 144.4 (CC=O), 149.7 (CHCHC=O), 198.3 (C=O).

3-Hydroxy-indan-1-one (B04) was prepared by refluxing 3-bromoindan-1-one (1,1 g, 5.2 mmol) for three hours in a mixture of water and acetone (10:7). The reaction mixture was then extracted with chloroform, evaporated and cleaned up by flash chromatography affording a yellow oil (0.25g, 1.6 mmol, 31%). IR ν_{max} (NaCl plate) 3418 (OH), 1714 (C=O) cm^{-1}. δ_H ($CDCl_3$) 2.50 (1H, dd, J_{gem}=19.1, J_{vic}=2.8, CH_2), 2.97 (1H, dd, J_{gem}=19.1, J_{vic}=7.0, CH_2), 4.60 (br, OH), 5.30 (1H, dd, J=2.8, 7.0, CHOH), 7.39 (1H, t, J=7.3, ArH), 7.58-7.61 (3H, m, ArH). *m/z* (gcms) 91 ($C_7H_7^+$, 100%), 119 (M^+-COH•, 32%), 120 (M^+-O=C••, 22%), 148 (M^+•, 66%), 149 (MH^+, 31%).

3-Methoxy-indan-1-one (B05) was obtained by extraction of a degraded solution of **C01** in water/methanol approx. 3:1. The extract was cleaned up by flash chromatography using hexane/ethyl acetate (9:1) as mobile phase affording an amber oil. IR ν_{max} (NaCl plate) 1718 (C=O) cm^{-1}. δ_H ($CDCl_3$) 2.68 (1H, dd, J_{gem}=18.6, J_{vic}=3.0, CH_2), 3.01 (1H, dd, J_{gem}=18.6, J_{vic}=6.5, CH_2), 3.51 (3H, s, OCH_3), 5.05 (1H, dd, J=3.0, 6.5, $CHOCH_3$), 7.50 (1H, m, ArH), 7.66-7.74 (2H, m, ArH), 7.78 (1H, d, J=8.0, CHCHCC=O). δ_C ($CDCl_3$) 43.4 (CH_2), 57.1 (CH_3), 76.7 ($CHOCH_3$), 123.2 (ArCH), 126.4 (ArCH), 129.5 (ArCH), 134.9 (ArCH), 136.8 ($CCHOCH_3$), 153.1, (CC=O), 202.8 (C=O). *m/z* (gcms) 77 ($C_6H_5^+$, 26%), 103 (M^+-C_3H_5O•, 70%), 162 (M^+, 100%).

Acetic acid 4-(3-oxo-butyl)-phenyl ester (B07) was prepared from 4-(4-Hydroxy-phenyl)-butan-2-one (3.3 g, 20 mmol), acetic anhydride (1.9 ml, 20 mmol) and triethylamine (2.9 ml, 20 mmol) in dry DCM at 0°C for one hour. The reaction mixture was washed with 0.1 M HCl solution and 0.01 M potassium hydrogen carbonate solution before evaporation of the solvent. IR ν_{max} (NaCl plate) 1760, 1715 (C=O) 1196 (C-O) cm^{-1}. δ_H ($CDCl_3$) 2.10 (3H, s, CH_3C=O), 2.25 (3H, s, CH_3COO), 2.72 (2H, t, J=7.5, CH_2C=O), 2.85 (2H, t, J=7.5, CH_2CH_2C=O), 6.97 (2H, d, J=8.0, CH_3OCCH), 7.16 (2H, d, J=8.0, $CH_3OCCHCH$). δ_C ($CDCl_3$) 20.8 (CH_3COO), 28.9 (CH_2CH_2C=O), 29.7 (CH_3C=O), 44.8 (CH_2C=O), 121.3 (2C, ArCH), 129.0 (2C, ArCH) 138.3 ($CH_3OCCHCHC$) 148.7 (CH_3OOC), 169.3 (CH_3COO), 207.4 (C=O). *m/z* (gcms) 107 (100%), 164 (M^+-CH_3CO•, 85%), 189 (43%)

Acetic acid 4-(1-bromo-3-oxo-butyl)-phenyl ester (B08) was prepared from **B07** according to General Procedure 1. A NMR of the reaction mixture (CCl_4) confirmed the presence of the product but it degraded during evaporation of the solvent. δ_H (CCl_4) 2.11 (3H, s, CH_3C=O), 2.29 (3H, s, CH_3COO), 3.11 (H, dd, J_{gem}=14.5, J_{vic}=7.5, CH_2C=O),

3.41 (2H, dd, J_{gem}=14.5, J_{vic}=7.5, $C\underline{H}_2C=O$), 4.44 (H, t, J=7.5, $C\underline{H}Br$), 6.97 (2H, d, J=8.5, $CH_3OCC\underline{H}$), 7.17 (2H, d, J=8.15, $CH_3OCCHC\underline{H}$).

4-(4-Trimethylsilanyloxy-phenyl)-butan-2-one (B09) was prepared from 4-(4-Hydroxy-phenyl)-butan-2-one (3.3 g, 20 mmol), TMS (2.6 ml, 22 mmol) triethylamine (3.1 ml, 22 mmol) in DCM. The reaction mixture was washed with 0.01 M potassium hydrogen carbonate solution before evaporation of the solvent. IR ν_{max} (NaCl plate) 1716 (C=O) cm^{-1}. *m/z* (gcms) 180 (M^+-C_3H_4O, 100%), 236 (M^+, 45%).

4-(4-Methoxy-phenyl)-butan-2-one (B10) was prepared from 4-(4-Hydroxy-phenyl)-butan-2-one (1.64 g, 10 mmol) and methyl iodide (1.56 g, 11 mmol) in methanol in the presence of sodium hydroxide (24 mmol, 4 ml of an aqueous 6M solution) at room temperature overnight. The compound was extracted with DCM and, after evaporation, the product was obtained in the form of a yellow oil (0.4 g, 2.24 mmol, 22%). IR ν_{max} (NaCl plate) 1715 (C=O), 1245 (C-O) cm^{-1}. δ_H ($CDCl_3$) 1.98 (3H, s, $C\underline{H}_3C=O$), 2.58 (2H, t, J=7.5, $C\underline{H}_2C=O$), 2.72 (2H, t, J=7.5, $C\underline{H}_2CH_2C=O$), 3.63 (3H, s, $C\underline{H}_3O$), 6.73 (2H, d, J=9.0, $CH_3OCC\underline{H}$), 7.00 (2H, d, J=9.0, $CH_3OCCHC\underline{H}$). δ_C ($CDCl_3$) 28.2 ($\underline{C}H_2CH_2C=O$), 29.1 ($\underline{C}H_3C=O$), 44.5 ($\underline{C}H_2C=O$), 54.4 ($\underline{C}H_3O$), 113.3 (2C, Ar$\underline{C}$H), 128.6 (2C, Ar$\underline{C}$H) 132.4 ($CH_3OCCHC\underline{C}$) 157.4 ($CH_3O\underline{C}$) 207.0 ($\underline{C}$=O). *m/z* (gcms) 121 (M^+-C_3H_5O•, 100%), 178 (M^+, 75%).

4-Bromo-4-(4-trimethylsilanyloxy-phenyl)-butan-2-one (B13) *m/z* (gcms) 115 (100%), 299 (M^+-CH_3, $C_8H_8BrO^+$, 88%), 299 (M^+-CH_3 (^{79}Br), 80%), 314 (M^+, 68%), 316 (M^+(^{79}Br), 65%).

4-Bromo-4-(4-methoxy-phenyl)-butan-2-one (B14) *m/z* (gcms) 199 ($C_8H_8BrO^+$, 100%),199 ($C_8H_8BrO^+$ (^{79}Br), 93%), 256 (M^+, 81%), 258 (M^+(^{79}Br), 77%).

4b,4c,9b,9c-Tetrahydro-cyclobuta[1,2-a;3,4-a']diindene-5,10-dione and **4b,4c,9a,9b-Tetrahydro-cyclobuta[1,2-a;4,3-a']diindene-9,10-dione (B15)** result from photodegradation of 2-Inden-1-one. After 2 month in a clear glass vial at room temperature, a sample of 2-inden-1-one degraded afford white crystals. IR ν_{max} (KBr) 1718, 1703 (C=O). δ_H ($CDCl_3$) 3.31 (1H, m), 4.65 (1H, d, J=8.0), 5.87 (1H, dd, J_1=3.3, J_2=9.5), 6.42 (1H, dd, J_1=3.0, J_2=9.5), 7.10 (1H, d, J=7.6), 7.28 (1H, m), 7.36-7.43 (2H, m), 7.48-7.55 (2H, m), 7.59 (1H, m), 7.84 (1H, d, J=7.5). δ_C ($CDCl_3$) 41.2 (CH), 51.0 (CH), 124.0 (CH), 124.3 (CH), 125.4 (Ar$\underline{C}$H), 127.5 (Ar$\underline{C}$H), 127.7 (Ar$\underline{C}$H), 127.8 (Ar$\underline{C}$H), 127.8 (Ar$\underline{C}$H), 127.9 (Ar$\underline{C}$H), 129.0 (Ar$\underline{C}$H), 131.4 ($\underline{C}$), 133.1 ($\underline{C}$), 134.5 ($\underline{C}$), 135.1 (Ar$\underline{C}$H), 155.9 ($\underline{C}C=O$), 205.8 ($\underline{C}$=O). *m/z* (gcms, major peak) 232 (100%)

Etilevodopa, 2-Amino-3-(3,4-dihydroxy-phenyl)-propionic acid ethyl ester hydrochloride salt (B16) was prepared from L-dopa by one of two procedures. A)[322] To a solution of L-dopa (0.39 g, 2 mmol) in 100 ml of ethanol at -40°C was added thionyl chloride (2.2 mmol), and the reaction mixture was stirred for 2 days at room temperature. The solvent was evaporated and a colourless oil was obtained. B) A solution of L-dopa (0.39 g, 2 mmol) in 100 ml of ethanol was bubbled with gaseous HCl until the L-Dopa was completely dissolved and the reaction mixture was stirred for 2 days at room temperature. The solvent was evaporated and a colourless oil was obtained. In order to eliminate residual HCl, the product was further flushed with air overnight. IR ν_{max} (NaCl plate) 3235 (OH), 1736 (C=O) cm^{-1} δ_H ($(CD_3)_2SO$) 1.10 (3H, $C\underline{H}_3$), 2.88 (1H, dd, J_{gem}=14.0, J_{vic}=7.5, $CC\underline{H}_2CH$), 3.02 (1H, dd, J_{gem}=14.0, J_{vic}=5.5, $CC\underline{H}_2CH$), 4.00-4.12 (3H, m, $C\underline{H}C=O$, $CH_3C\underline{H}_2OC=O$), 6.44 (1H, dd, J=1.5, 8.0, $CC\underline{H}CHCOH$), 6.60 (1H, d, J=2.0, $CCHC\underline{H}COH$), 6.67 (1H, d, J=8.0, $CC\underline{H}COH$). δ_C ($(CD_3)_2SO$) 14.3 ($\underline{C}H_3$), 35.9 ($C\underline{C}H_2CH$), 54.0 ($CCH_2\underline{C}H$), 62.1 ($CH_3\underline{C}H_2$), 116.3 ($C\underline{C}HCOH$), 117.3 ($CCH\underline{C}HCOH$), 120.7 ($C\underline{C}HCHCOH$), 125.4 ($CH_2\underline{C}$), 145.1 ($CCHCH\underline{C}OH$), 145.7 ($CCH\underline{C}OH$), 169.5 (C=O). *m/z* 226.1086 (M(-Cl), expected: 226.1079).

General Procedure 2: <u>preparation of 3-aminoindan-1-ones, 3-amino-2-methyl-indan-1-ones and 3-amino methyl propionates</u>

These compounds were prepared from 3-bromo-indan-1-one, 3-bromo-2-methyl-indan-1-one or 3-bromo-(methyl propionate) (1 mmol eq) in dry DCM (20 ml) to which was added triethylamine (1.9 mmol eq) and the appropriate amine (1 mmol eq). When the hydrochloride form of the amine was used, the quantity of triethylamine was doubled. Each reaction was stirred at 0°C for 3-5h unless otherwise stated. The solvent was removed by evaporation and the residue was cleaned up by flash column chromatography (silica gel, hexane/ethyl acetate 4:1 or DCM/methanol 95:5). The fractions containing the products were evaporated under reduced pressure and when possible, the residues obtained were recrystalised.

3-(Indan-1-ylamino)-indan-1-one (C01) was prepared from 1-aminoindane (0.13 g, 1 mmol) and 3-bromoindan-1-one (0.21 g, 1 mmol) as described in General Procedure 2. The product was cleaned up with hexane/ethyl acetate (8:2) and the oil obtained recrystalised from boiling hexane affording off-white needle crystals (0.19 g, 0.72 mmol) (72%). With the same mobile phase, it was possible to separate two pairs of enantiomers.

It was also possible to isolate the individual compounds by using an optically pure 1-aminoindane instead of the racemic one. In this way, a pair of two diastereomers was produced and the individual compounds were separated as described before.

3*S*-(Indan-1*R*-ylamino)-indan-1-one (*R,S*-C01) and **3*R*-(Indan-1*R*-ylamino)-indan-1-one (*R,R*-C01)** were prepared using 1*R*-aminoindane.

3*S*-(Indan-1*S*-ylamino)-indan-1-one (*S,S*-C01) and **3*R*-(Indan-1*S*-ylamino)-indan-1-one (*S,R*-C01)** were prepared from 1*S*-aminoindane.

3*S*-(Indan-1*R*-ylamino)-indan-1-one (*R,S*-C01) and **3*R*-(Indan-1*S*-ylamino)-indan-1-one (*S,R*-C01)** share the same chemical characteristics as they are enantiomers. MP 128-130°C, IR ν_{max} (KBr) 1710 (C=O) cm^{-1}. δ_H ($CDCl_3$) 1.63 (1H, br, s, NH), 1.86, 2.50 (2x1H, m, $NHCHCH_2CH_2$), 2.62 (1H, dd, J_{gem}=18.6, J_{vic}=3.5, $CHCH_2C=O$), 2.86, 3.06 (2x1H, m, $NHCHCH_2CH_2$), 3.12 (1H, dd, J_{gem}=18.6, J_{vic}=7.0, $CHCH_2C=O$), 4.46 (1H, t, J=7.0 $NHCHCH_2CH_2$), 4.68 (1H, dd, J=3.5, 6.5, $CHCH_2C=O$), 7.26 (3H, m, ArH), 7.47 (2H, m, ArH), 7.67 (1H, m, ArH), 7.77 (2H, m, ArH), δ_C ($CDCl_3$) 30.5 (CH_2CH_2CH), 36.0 (CH_2CH_2CH), 46.7 ($CHCH_2C=O$), 55.1 ($CHCH_2C=O$), 62.5 (CH_2CH_2CH), 123.3 (ArCH), 124.1 (ArCH), 124.9 (ArCH), 126.0 (ArCH), 126.3 (ArCH), 127.6 (2C, ArCH), 128.6 (ArCH), 134.8 (C), 136.6 (C), 143.4 (2C), 204.6 (C=O). *m/z* 264.1399 (MH^+, expected: 264.1388).

3*R*-(Indan-1*R*-ylamino)-indan-1-one (*R,R*-C01) and **3*S*-(Indan-1*S*-ylamino)-indan-1-one (*S,S*- C01)** share the same chemical characteristics as they are enantiomers. MP 88-90°C. IR ν_{max} (KBr) 1710 (C=O) cm^{-1}. δ_H ($CDCl_3$) 1.69 (1H, br, s, NH), 2.01, 2.57 (2x1H, m, $NHCHCH_2CH_2$), 2.68 (1H, dd, J_{gem}=18.6, J_{vic}=3.0, $CHCH_2C=O$), 2.91 (1H, m, $NHCHCH_2CH_2$), 3.10 (1H, dd, J_{gem}=18.6, J_{vic}=6.5, $CHCH_2C=O$), 3.11 (1H, m, $NHCHCH_2CH_2$), 4.46 (1H, t, J=6.5, $NHCHCH_2CH_2$), 4.68 (1H, dd, J=3.0, 6.5, $CHCH_2C=O$), 7.25 (3H, m, ArH), 7.34 (1H, m, ArH), 7.47 (1H, m, ArH), 7.68 (2H, m, ArH), 7.80 (1H, d, J=7.5, ArH). δ_C ($CDCl_3$) 30.3 (CH_2CH_2CH), 34.2 (CH_2CH_2CH), 45.8 ($CHCH_2C=O$), 54.0 ($CHCH_2C=O$), 61.6 (CH_2CH_2CH), 123.4 (ArCH), 123.9, (ArCH), 124.8 (ArCH), 125.8 (ArCH), 126.5, 127.7 (ArCH), 128.7 (ArCH), 134.9 (ArCH), 136.9 (2C), 143.5 (2C), 204.7 (C=O). *m/z* 264.1406 (MH^+, expected: 264.1388).

(*R,S*)-3-(Indan-2-ylamino)-indan-1-one (C02) was prepared from 2-aminoindane hydrochloride as described in General Procedure 2. The product was cleaned up by flash chromatography with hexane/ethyl acetate and the oil obtained was recrystalised from boiling hexane affording off white needle crystals (40%). MP 88-90°C. IR ν_{max} (KBr) 1713 (C=O) cm^{-1}. δ_H ($CDCl_3$) 1.66 (1H, br, S, NH), 2.62 (1H, dd, J_{gem}=18.6, J_{vic}=3.0, $CHCH_2C=O$), 2.84, (1H, dd, J_{gem}=15.6, J_{vic}=6.5, $NCHCH_2Ar$), 2.94 (1H, dd, J_{gem}=15.6,

J_{vic}=6.5, NCHC$\underline{H}_2$Ar), 3.08 (1H, dd, J_{gem}=18.6, J_{vic}=6.5, CHC$\underline{H}_2$C=O), 3.22, (1H, dd, J_{gem}=15.6, J_{vic}=7.0, NCHC$\underline{H}_2$Ar), 3.28 (1H, dd, J_{gem}=15.6, J_{vic}=7.0, NCHC$\underline{H}_2$Ar), 3.86 (1H, qi, J=7.0, NHC$\underline{H}$CH$_2$Ar), 4.61 (1H, dd, J=3.0, 6.5, NHC$\underline{H}$CH$_2$C=O), 7.18-7.25 (4H, m, Ar$\underline{H}$), 7.47 (1H, t, J=7.6, Ar$\underline{H}$), 7.69 (2H, m, Ar$\underline{H}$), 7.78 (1H, d, J=8, CHCC=O). δ_C ($CDCl_3$) 40.1, 40.8 (2C CH$\underline{C}$H$_2$Ar), 45.6 (CH$\underline{C}$H$_2$C=O), 54.7, 58.3 (2C, CH$_2\underline{C}$HNH$\underline{C}$HCH$_2$C=O), 123.4 (Ar$\underline{C}$H), 124.6, 124.7 (2C, Ar$\underline{C}$H), 126.0 (Ar$\underline{C}$H), 126.6 (2C, Ar$\underline{C}$H), 128.8 (Ar$\underline{C}$H), 134.9 (Ar$\underline{C}$H), 136.7 ($\underline{C}$), 141.2, 141.4 (2$\underline{C}$), 155.5 ($\underline{C}$C=O), 204.5, ($\underline{C}$=O). *m/z* 264.1401 (MH^+, expected: 264.1388).

3-Dimethylamino-indan-1-one (C12) was prepared according to General Procedure 2 from dimethylamine hydrochloride (0.08 g, 1 mmol) and 3-bromoindanone (0.21 g, 1 mmol). The reaction mixture was cleaned up with hexane/ethyl acetate (8:2). It afforded an amber oil (75 mg, 0.43 mmol) in a yield of 43%. IR ν_{max} (NaCl plate) 1715 (C=O) cm^{-1}. δ_H ($CDCl_3$) 2.06 (6H, s, C$\underline{H}_3$NC$\underline{H}_3$), 2.61 (1H, dd, J_{gem}=19.0, J_{vic}=7.0, CHC$\underline{H}_2$C=O), 2.74 (1H, dd, J_{gem}=19.0, J_{vic}=3.0, CHC$\underline{H}_2$C=O), 4.61 (1H, dd, J=3.0, 7.0, C$\underline{H}$CH$_2$C=O), 7.47 (1H, t, J=7.5 C$\underline{H}$CHCHCC=O), 7.67 (1H, t, J= 7.5, C$\underline{H}$CHCC=O), 7.73 (1H, d, J= 7.5, C$\underline{H}$CHCHCHCC=O), 7.78 (1H, d, J= 7.5, C$\underline{H}$CC=O). δ_C ($CDCl_3$) 35.1 (CH$\underline{C}$H$_2$C=O), 40.5 (2C, $\underline{C}$H$_3$), 62.7 ($\underline{C}$HCH$_2$C=O), 123.2 ($\underline{C}$HCC=O), 126.7 ($\underline{C}$HCHCHCHCC=O), 128.8 ($\underline{C}$HCHCHCC=O), 134.8 ($\underline{C}$HCHCC=O), n.d. ($\underline{C}$CHCHCHCH$\underline{C}$C=O), 206.7 (C$\underline{C}$=O), *m/z* 176.1066 (MH^+, expected: 176.1075).

3-Ethylamino-indan-1-one (C13) was prepared as described before from ethylamine hydrochloride (0.16 g, 2 mmol) and 3-bromoindanone (0.42 g, 2 mmol). After evaporation, the residue of the reaction mixture was cleaned up with DCM/methanol (95:5) and a brown oil was afforded (0.14 g, 0.81 mmol) with 41 % yield. IR ν_{max} (NaCl plate) 1713 (C=O) cm^{-1}. δ_H ($CDCl_3$) 1.16 (3H, d, J=7.0, C$\underline{H}_3$), 1.68 (1H, br, S, N$\underline{H}$), 2.52 (1H, dd, J_{gem}=18.6, J_{vic}=3.0, CHC$\underline{H}_2$C=O), 2.76 (2H, m, C$\underline{H}_2$CH$_3$), 2.96 (1H, dd, J_{gem}=18.6, J_{vic}=6.5, CHC$\underline{H}_2$C=O), 4.47 (1H, dd, J=3.0, 6.5, NHC$\underline{H}$CH$_2$C=O), 7.42 (1H, t, J= 7.5, C$\underline{H}$CHCC=O), 7.61-7.68 (2H, m, C$\underline{H}$C$\underline{H}$CCHNH) 7.73 (1H, d , J= 7.5, C$\underline{H}$CC=O). δ_C ($CDCl_3$) 15.3 ($\underline{C}$H$_3$), 41.8 ($\underline{C}$H$_2$CH$_3$), 44.7 (CH$\underline{C}$H$_2$C=O), 56.0 (NH$\underline{C}$HCH$_2$), 123.2 ($\underline{C}$HCHCC=O), 125.8 ($\underline{C}$HCCHNH), 128.5 ($\underline{C}$HCC=O), 134.7 ($\underline{C}$HCHCCHNH), 136.7 ($\underline{C}$CHNH), 155.9 ($\underline{C}$C=O), 204.5 ($\underline{C}$=O). *m/z* 176.1070 (MH^+, expected: 176.1075).

3-(3-Ethylamino-indan-1-one)-indan-1-one (C13a) was obtained as another product of the synthesis of 3-ethylamino-indan-1-one (**C13**) A mixture of two diastereomers was obtained with approximately the same quantity of the two compounds (0.61 g 0.4 mM) 20%. IR ν_{max} (NaCl plate) 1713 (C=O) cm^{-1}. δ_H ($CDCl_3$) 0.95, 1.14 (3H, J= 7.0, C$\underline{H}_3$), 2.33-2.69 (6H, m, C$\underline{H}_2$CHN(C$\underline{H}_2$CH$_3$)CHC$\underline{H}_2$), 4.54, 4.66 (2H, dd, J=6.0, 5.0, dd, J=7.0,

3.5, C<u>H</u>N(CH_2CH_3)C<u>H</u>), 7.44 (2H, m, Ar<u>H</u>), 7.63-7.90 (6H, m, Ar<u>H</u>). δ_C ($CDCl_3$) 13.7, 14.5 (<u>C</u>H_3), 38.8, 40.1 (<u>C</u>H_2CH_3), 39.0, 40.3 (2C, CH<u>C</u>H_2C=O), 55.3, 57.1 (2C, N<u>C</u>HCH_2), 122.2, 122.4 (2C, Ar<u>C</u>H), 125.4, 126.0 (2C, Ar<u>C</u>H), 128.0, 128.1 (2C, Ar<u>C</u>H), 134.1, 134.4 (2C, Ar<u>C</u>H), 136.2, 136.4 (2C, <u>C</u>CHN), 155.2, 155.4 (2C, <u>C</u>C=O), 203.1, 203.5 (2C, <u>C</u>=O). *m/z* 306.1502 (MH^+, expected: 306.1494).

3-Piperidin-1-yl-indan-1-one (C14) was prepared as described in General Procedure 2 from 3-bromoindan-1-one (0.21 g, 1 mmol) and piperidine (0.08 g, 1 mmol). The product was cleaned up by flash chromatography with hexane/ethyl acetate (8:2). A brown oil was afforded (0.18 g, 0.84 mmol), 84%. IR ν_{max} (NaCl plate) 1715 (C=O), 2801, 2853, 2933 (aliphatic C-C) cm^{-1}. δ_H ($CDCl_3$) 1.45 (2H, m, $NCH_2CH_2C\underline{H}_2$), 1.56 (4H, m, $NCH_2\underline{C}H_2$), 2.30 (2H, m, $NHC\underline{H}_2$), 2.47 (2H, m, $NHC\underline{H}_2$), 2.61 (1H, dd, J_{gem}=18.8, J_{vic}=7.0, $CHC\underline{H}_2C$=O), 2.77 (1H, dd, J_{gem}=18.8, J_{vic}=3.5, $CHC\underline{H}_2C$=O), 4.53 (1H, dd, J=3.5, 7.0, NC<u>H</u>), 7.42 (1H, t, J=7.52, C<u>H</u>CHCHCC=O), 7.63 (1H, t, J=7.52, C<u>H</u>CHCC=O), 7.73 (2H, m, C<u>H</u>CHCHC<u>H</u>CC=O). δ_C ($CDCl_3$) 24.5 ($NCH_2CH_2\underline{C}H_2$), 26.2 (2C, $NCH_2\underline{C}H_2$), 36.4 (2C, $N\underline{C}H_2CH_2$), 49.7 ($CH\underline{C}H_2C$=O), 63.2 (N<u>C</u>H), 123.0 (<u>C</u>HCC=O), 126.7 (<u>C</u>HCHCHCHCC=O), 128.4 (<u>C</u>HCHCHCC=O), 134.5 (<u>C</u>HCHCC=O), 137.5 (<u>C</u>CHCHCHCHCC=O), 154.7 (<u>C</u>C=O), 204.7 (<u>C</u>=O). *m/z* 216.1393 (MH^+, expected: 216.1388).

3-Phenethylamino-indan-1-one (C15) was prepared from phenylethylamine (0.12g, 1mmol) and 3-bromoindan-1-one (0.21 g, 1 mmol) according to General Procedure 2. The product was cleaned up with hexane/ethyl acetate (8:2) and a brow oil was obtained with a yield of 36% (0.09 g, 0.36 mmol). IR ν_{max} (NaCl plate) 1712 cm^{-1}. δ_H ($CDCl_3$) 2.50 (1H, dd, J_{gem}=18.8, J_{vic}=3.2, $NHCHC\underline{H}_2C$=O), 2.84-3.05 (5H, $NHCHC\underline{H}_2C$=O, $NHC\underline{H}_2C\underline{H}_2$), 4.48 (1H, dd, J=3.2, 6.8, $NHC\underline{H}CH_2C$=O), 7.22-7.28 (3H, m, CC<u>H</u>CHC<u>H</u>CHC<u>H</u>), 7.30 (2H, m, CCHC<u>H</u>CHC<u>H</u>CH), 7.43 (1H, t, J=7.3, C<u>H</u>CHCHCC=O), 7.57-7.65 (2H, m, C<u>H</u>CHC<u>H</u>CHCC=O), 7.74 (1H, d, J=7.5, C<u>H</u>CC=O). δ_C ($CDCl_3$) 36.3 ($NCH\underline{C}H_2$), 44.4 ($NCH_2\underline{C}H_2$), 48.3 ($N\underline{C}H_2CH_2$), 55.8 (N<u>C</u>HCH$_2$), 123.2 (<u>C</u>HCC=O), 125.7 (<u>C</u>HCHCHCCH_2CH_2), 126.2 (<u>C</u>HCHCHCHCC=O), 128.4 (3C, <u>C</u>HCHCCH_2CH_2 & <u>C</u>HCHCHCC=O), 128.6 (2C, <u>C</u>HCCH_2CH_2), 134.8 (<u>C</u>HCHCC=O), 136.7 (<u>C</u>CH_2CH_2), 139.4 (<u>C</u>CHCHCHCHCC=O), 155.5 (<u>C</u>C=O), 204.4 (<u>C</u>=O). *m/z* 252.1386 (MH^+, expected: 252.1388).

3-Atenolol-indan-1-one, 2-(4-{2-Hydroxy-3-[isopropyl-(3-oxo-indan-1-yl)-amino]-propoxy}-phenyl)-acetamide (C16) was prepared from atenolol (0.27 g, 1 mmol) and 3-bromoindanone (0.21 g, 1 mmol) according to General Procedure 2. The evaporated residue was cleaned up by flash chromatography using DCM/methanol (92.5:7.5). Two

spots were observed by TLC and were separated during the clean up procedure. However, only the diastereomer firstly eluted was obtained uncontaminated with the other according to the NMR. The second was apparently a mixture of the two isomers.

The following data corresponds to the diastereomer obtained uncontaminated (0.094 g, 0.24 mmol, 11.8%). IR ν_{max} (NaCl plate) 1670, 1707, (C=O) 3354 (NH_2) cm^{-1}. δ_H ($CDCl_3$) 1.13 (3H, d, J=6.4, C$\underline{H}_3$), 1.25 (3H, d, J=6.4, C$\underline{H}_3$), 2.44 (1H, dd, J_{gem}=13.6, J_{vic}=6.1, NC$\underline{H}_2$CH), 2.63 (1H, dd, J_{gem}=13.6, J_{vic}=6.1, NC$\underline{H}_2$CH), 2.70 (1H, dd, J_{gem}=19.1, J_{vic}=3.4, CHC$\underline{H}_2$C=O), 2.77 (1H, dd, J_{gem}=19.1, J_{vic}=6.8, CHC$\underline{H}_2$C=O), 3.14 (1H, m, $CH_3C\underline{H}CH_3$), 3.52 (2H, s, C$\underline{H}_2$C=ONH_2), 3.66 (2H, m, CHC$\underline{H}_2$O), 3.90 (1H, m, C$\underline{H}$OH), 4.70 (1H, dd, J=3.4, 6.8, NC$\underline{H}CH_2$C=O), 5.48, (1H, s, br) 5.72 (1H, s, br), 6.75 (2H, d, J=8.9, OCCHC$\underline{H}$CCH_2), 7.16 (2H, d, J=8.9, OCC$\underline{H}$CHCCH_2), 7.40 (1H, t, J=7.5, C$\underline{H}$CHCC=O), 7.57 (1H, m, C$\underline{H}$CHCHCC=O), 7.65 (1H, d, J=4.1, C$\underline{H}$CHCHCHCC=O), 7.73 (1H, d, J=7.5, C$\underline{H}$CC=O). *m/z* 397.2121 (MH^+, expected: 397.2127).

3-(Tryptophan methyl ester)-indan-1-one, 3-(1H-Indol-3-yl)-2-(3-oxo-indan-1-ylamino)-propionic acid methyl ester (C17) was prepared from tryptophan methyl ester hydrochloride (0.50 g, 2 mmol) and 3-bromoindan-1-one (0.42 g, 2 mmol) as described in General Procedure 2. A racemic mixture of two diastereomers was obtained and they were not separated. The product was cleaned up with hexane/ethyl acetate (6:4) and an amber oil was obtained with a yield of 87% (0.61 g, 0.87 mmol). IR ν_{max} (NaCl plate) 3.040 (indole), 1714 (C=O) cm^{-1}. δ_H ($CDCl_3$) 2.22 (1H, br, s, N$\underline{H}$CHCOOCH3), 2.35, (1H, dd, J_{gem}=18.6, J_{vic}=3.0, NHCHC$\underline{H}_2$C=O), 2.48 (1H, dd, J_{gem}=18.6, J_{vic}=3.0, NHCHC$\underline{H}_2$C=O), 2.87* (1H, dd, J_{gem}=18.6, J_{vic}=6.5, NHCHC$\underline{H}_2$C=O), 2.91* (1H, dd, J_{gem}=18.6, J_{vic}=6.5, NHCHC$\underline{H}_2$C=O), 3.14-3.33 (2H, m, CC$\underline{H}_2$CHNH), 3.68, 3.77 (3H, 2s, C$\underline{H}_3$), 3.80, 3.89 (1H, 2m, NHC$\underline{H}$COOC$\underline{H}_3$), 4.39 (1H, m, NHC$\underline{H}CH_2$C=O), 7.00, 7.06 (1H, dd, J=1.5, J=25.0 CNHC$\underline{H}$C), 7.13-7.26, (2H, Ar$\underline{H}$), 7.28-7.43 (2H, Ar$\underline{H}$), 7.48 7.46-7.70 (3H, m, Ar$\underline{H}$), 8.55, 8.62 (1H, d, J=25.0, CN$\underline{H}$CH). δ_C ($CDCl_3$) 29.5, 29.6 (C$\underline{C}H_2$CHNH), 44.7, 45.2 (O=C$\underline{C}H_2$CHNH), 51.8, 51.8 ($\underline{C}H_3$), 54.6, 55.4 (O=CC$H_2\underline{C}$HNH), 60.2, 60.8 (CC$H_2\underline{C}$HNH), 110.5, 110.6 (CHC$\underline{C}$CHNH), 111.2 (Ar$\underline{C}$H), 118.3, 118.5 (Ar$\underline{C}$H), 119.2, 119.2 (Ar$\underline{C}$H), 121.9 (Ar$\underline{C}$H), 122.9, 123.0 (Ar$\underline{C}$H), 123.0, 123.1 (Ar$\underline{C}$H), 125.5 126.0 (Ar$\underline{C}$H), 127.1, 127.3 ($\underline{C}$), 128.5, 128.6 (Ar$\underline{C}$H), 134.5, 134.8 (Ar$\underline{C}$H), 136.1 ($\underline{C}$), 136.4, 136.5 ($\underline{C}$), 155.2, 155.3 (CH$\underline{C}$CHCH_2C=O), 175.3, 175.4 (O=$\underline{C}$OCH_3) 204.2, 204.3 ($\underline{C}$=O). *m/z* 349.1563 (MH^+, expected: 349.1552).

3-(Alanine methyl ester)-indan-1-one, 2-(3-Oxo-indan-1-ylamino)-propionic acid methyl ester (C18) was prepared from alanine methyl ester (0.279 g, 2 mmol) and 3-bromoindanone (0.42 g, 2 mmol) as described in General Procedure 2. The mobile

phase for the clean up procedure consisted of hexane/ethyl acetate (2:1). An amber oil was obtained (0.12 g, 0.50 mmol) as a racemic mixture of two diastereomers that were not separated. 25%. IR ν_{max} (NaCl plate) 1715 (C=O) cm^{-1}. δ_H ($CDCl_3$) 1.18, (3H, d, J_{vic}=4, $CHC\underline{H}_3$) 1.22* (3H, d, J_{vic}=4, $CHC\underline{H}_3$), 2.11 (1H, br, s, N$\underline{H}$), 2.42 (1H, dd, J_{gem}=18.7, J_{vic}=3.5, $C\underline{H}_2$), 2.44 (1H, dd, J_{gem}=18.7, J_{vic}=2.9, $C\underline{H}_2$), 2.85* (1H, dd, J_{gem}=18.7, J_{vic}=6.4, $C\underline{H}_2$), 2.92* (1H, dd, J_{gem}=18.7, J_{vic}=7.0, $C\underline{H}_2$), 3.45 (1H, q, J=7.0, $CH_3C\underline{H}NH$), 3.55* (1H, q, J=7.0, $CH_3C\underline{H}NH$), 4.31 (1H, dd, J=2.9, 6.4, $C\underline{H}CH_2C{=}O$), 4.41* (1H, dd, J=3.5, 7.0, $C\underline{H}CH_2C{=}O$), 7.41 (1H, m, $C\underline{H}CHCHCC{=}O$), 7.63 (3H, m, $C\underline{H}CHC\underline{H}C\underline{H}CC{=}O$), δ_C ($CDCl_3$) 19.2, 19.7 ($\underline{C}H_3CH$), 44.9, 45.4 ($\underline{C}H_2$), 52.0 ($O\underline{C}H_3$), 54.3 ($N\underline{C}HCH_2$), 54.7, 55.1 ($N\underline{C}HCH_3$), 123.2, 123.4 ($\underline{C}HCC{=}O$), 125.4, 126.2 ($\underline{C}HCHCHCHCC{=}O$), 128.7, 128.8 ($\underline{C}HCHCHCC{=}O$), 134.7, 135.0 ($\underline{C}HCHCC{=}O$), 136.55, 136.6 ($\underline{C}CHCHCHCHCC{=}O$), 155.2, 155.4 ($\underline{C}C{=}O$), 176.0, 176.2 ($\underline{C}OOCH_3$), 204.0, 204.2 ($\underline{C}{=}O$). *m/z* 234.1135 ($MH^+$, expected: 234.1130).

3-(Phenylalanine benzyl ester indanone), 2-(3-Oxo-indan-1-ylamino)-3-phenyl-propionic acid benzyl ester (C19) was prepared from phenylalanine benzyl ester *p*-toluenesulfonate salt (0.43 g, 1 mmol) and 3-bromoindanone (0.21 g, 1 mmol) according to General Procedure 2. Hexane/ethyl acetate (8:2) was used as mobile phase for the clean-up procedure. A brown oil was obtained (0.16 g, 0.42 mmol), with a yield of 42%. A mixture of two diastereomers was obtained (22:78) the data corresponds to the predominant compound only. IR ν_{max} (NaCl plate) 1715 (C=O) cm^{-1}. δ_H ($CDCl_3$) 2.38 (1H, dd, J_{gem}=18.6, J_{vic}=3.5, $NHCHC\underline{H}_2C{=}O$), 2.86 (1H, dd, J_{gem}=18.6, J_{vic}=6.8, $NHCHC\underline{H}_2C{=}O$), 2.99 (1H, dd, J_{gem}=13.5, J_{vic}=7.0, $NHCHC\underline{H}_2ph$), 3.06 (1H, dd, J_{gem}=13.5, J_{vic}=6.5, $NHCHC\underline{H}_2ph$), 3.74 (1H, t, J=7.0, $NHCHC\underline{H}_2ph$), 4.36 (1H, dd, J=3.5, 6.8, $C\underline{H}CH_2C{=}O$), 5.09 (2H, s, $OC\underline{H}_2ph$), 7.20-7.61 (13H, m, Ar$\underline{H}$), 7.72 (1H, d, J=7.5, $C\underline{H}CC{=}O$). δ_C ($CDCl_3$) 40.2 ($ph\underline{C}H_2CH$), 45.4 ($CH\underline{C}H_2C{=}O$), 55.7 ($\underline{C}HCH_2C{=}O$), 61.8 ($phCH_2\underline{C}H$), 66.9 ($ph\underline{C}H_2O$), 123.4 ($\underline{C}HCHCHCCH_2CH$), 126.3 ($\underline{C}HCHCC{=}O$), 126.8 ($\underline{C}HCHCHCCH_2O$), 128.4 (2C, $CH\underline{C}HCCH_2O$), 128.5 ($\underline{C}HCHCHCHCC{=}O$), 128.5 (2C, $\underline{C}HCCH_2CH$), 128.6 (2C, $\underline{C}HCHCCH_2CH$), 128.7 ($\underline{C}HCC{=}O$), 129.3 (2C, $\underline{C}HCHCCH_2O$), 134.6 ($\underline{C}HCHCHCC{=}O$), 136.7 ($CH\underline{C}C{=}O$), 137.1 ($\underline{C}CHCHCHCHCC{=}O$), 155.3 ($CH\underline{C}CH_2CH$), 167.2 ($CH\underline{C}CH_2O$), 174.7 ($\underline{C}OOCH_2$), 203.9 ($C\underline{C}{=}O$). *m/z* 386.1776 ($MH^+$, expected: 385.1678).

3-Desloratadine-indan-1-one, 3-[4-(8-Chloro-5,6-dihydro-benzo[5,6]cyclohepta[1,2-b]pyridin-11-ylidene)-piperidin-1-yl]-indan-1-one (C20) was prepared from desloratadine (0.31 g, 1 mmol) and 3-bromoindan-1-one (0.21 g, 1 mmol) as described in General Procedure 2. After evaporation, the reaction mixture residue was cleaned up by flash column chromatography, using DCM/methanol (96:4). A reddish crystalline solid

was obtained (0.38 g, 0.86 mmol) 86%. IR ν_{max} (KBr) 1710 (C=O) cm^{-1}. δ_H ($CDCl_3$), 2.27-2.50 (6H, m, $C\underline{H}_2$), 2.5-2.77 (2H, m, $C\underline{H}_2$) 2.79-2.96 (4H, m, $C\underline{H}_2$), 3.31-3.42 (2H, m, $C\underline{H}_2$), 4.62 (1H, m, $CH_2N\underline{C}H$), 7.05-7.15 (4H, m, Ar$\underline{C}$H), 7.41-7.45 (2H, m, Ar$\underline{C}$H), 7.65 (1H, t, J=7.9, Ar$\underline{C}$H), 7.72-7.77 (2H, m, Ar$\underline{C}$H), 8.38 (1H, m, Ar$\underline{C}$H). δ_C ($CDCl_3$) 31.3, 31.7 (2C, $C\underline{C}H_2\underline{C}H_2C$), 36.1, 36.3 (2C, $\underline{C}H_2C\underline{C}H_2$), 45.8 (NCH$\underline{C}H_2$), 48.5, 48.6 (2C, N$\underline{C}H_2$), 62.5 (N$\underline{C}HCH_2$), 122.1 (Ar$\underline{C}$H), 123.1 (Ar$\underline{C}$H), 125.9 (Ar$\underline{C}$H), 126.7 (Ar$\underline{C}$H), 128.7 (Ar$\underline{C}$H), 128.9 (Ar$\underline{C}$H), 130.7 (Ar$\underline{C}$H), 132.6 ($\underline{C}$), 133.0 ($\underline{C}$), 133.4 ($\underline{C}$), 134.8 (Ar$\underline{C}$H), 137.3 (Ar$\underline{C}$H), 137.5 ($\underline{C}$), 138.3 ($\underline{C}$), 139.4 ($\underline{C}$), 146.5 (Ar-N$\underline{C}$H), 157.3 ($\underline{C}$), 175.0 ($\underline{C}$C=O), 207.7 ($\underline{C}$=O). *m/z* 441.1750 ($MH^+$, expected: 441.1734).

3-ephedrin-indan-1-one, 3-[(2-Hydroxy-1-methyl-2-phenyl-ethyl)-methyl-amino]-indan-1-one (C21) was prepared from ephedrine (0.21 g, 1 mmol) and 3-bromoindan-1-one (0.21 g, 1 mmol) as described in General Procedure 2. Ephedrine has two chiral centres but consists in only one of the diastereomers (*R,S*; *S,R*). Therefore, the product of the reaction consists in four isomers consisting in two pairs of enantiomers (*R,S,S*; *R,S,R*; *S,R,S*; *S,R,R*). The product was purified by flash chromatography using hexane/ethyl acetate (8:2) and the diastereomers were not separated (0.12 g, 0.41 mmol) 41%. After a few days, the mixture solidified affording a yellow solid. IR ν_{max} (KBr) 3429 (OH), 1692 (C=O) cm^{-1}. δ_H ($CDCl_3$) 1.06, 1.10 (3H, 2d, J=7.0, $C\underline{H}_3CH$), 1.91, 2.16 (3H, 2s, $NC\underline{H}_3$) 2.52-2.75 (2H, m, $C\underline{H}_2$), 2.92, 3.14 (1H, 2m, NC$\underline{H}CH_3$), 4.74 (1H, m, NC$\underline{H}CH_2$), 4.87, 4.95 (1H, 2d, J=5.5, J=4.5). δ_C ($CDCl_3$) 11.8, 12.0 ($\underline{C}H_3CH$), 30.1 33.8 ($N\underline{C}H_3$) 37.2, 37.5 ($\underline{C}H_2$), 60.7 ($CH_2\underline{C}HN$), 63.6, 64.0 ($CH_3\underline{C}HN$) 73.8, 74.1 ($\underline{C}$HOH), 123.0, 123.1 ($\underline{C}$HCHCHCCHOH), 126.0 126.2 (2C, $\underline{C}$HCCHOH) 126.1, 126.2 ($\underline{C}$HCHCC=O), 127.1, 127.2 ($\underline{C}$HCC=O), 128.1, 128.2 (2C, $\underline{C}$HCHCCHOH), 128.5, 128.6 ($\underline{C}$HCHCHCHCC=O), 134.8, 134.9 ($\underline{C}$HCHCHCC=O), 137.1, 137.2 ($\underline{C}$CHCHCHCHCC=O), 142.0, 142.8 ($\underline{C}$CHOH), 155.5, 155.6 ($\underline{C}$C=O), 204 (C=O) *m/z* 296.1646 (MH^+, expected: 296.1651).

2-Methyl-3-piperidin-1-yl-indan-1-one (C22) was prepared as described in General Procedure 2, using 3-bromo-2-methyl-indan-1-one (0.22 g, 1 mmol) and piperidine (85 mg, 1 mmol). A brown oil was obtained (78 mg, 0.34 mmol) after clean up by flash chromatography using hexane/ethyl acetate (8:2) 34%. IR ν_{max} (NaCl plate) 1715 (C=O) cm^{-1}. δ_H ($CDCl_3$) 1.37 (3H, d, J=7.5, $C\underline{H}_3$), 1.47 (2H, m, $NCH_2CH_2C\underline{H}_2$), 1.60 (4H, m, $NCH_2C\underline{H}_2$), 2.43 (2H, m, $NHC\underline{H}_2$), 2.53 (2H, m, $NHC\underline{H}_2$), 2.79 (1H, m, $CH_3C\underline{H}C$=O), 4.09 (1H, d, NC$\underline{H}$), 7.44 (1H, t, J=7.52, C$\underline{H}$CHCHCC=O), 7.65 (1H, m, C$\underline{H}$CHCHCHCC=O), 7.71 (2H, m, C$\underline{H}$C$\underline{H}$CC=O). δ_C ($CDCl_3$) 16.4 (CH_3), 24.6 ($\underline{C}H_2CH_2CH_2N$), 26.4 (2C, $\underline{C}H_2CH_2N$), 41.8 ($\underline{C}HCH_3$), 50.3 (2C, $\underline{C}H_2N$), 72.1 ($\underline{C}$HN), 123.3 ($\underline{C}$HCC=O), 126.5 ($\underline{C}$HCHCHCHCC=O), 128.5 ($\underline{C}$HCHCHCC=O), 134.7 ($\underline{C}$HCHCC=O), 136.6

(CCHCHCHCHCC=O), 153.4 (CC=O), 207.3 (C=O). *m/z* 230.1541 (MH^+, expected: 230.1545).

3-Desloratadine]-2-methyl-indan-1-one, 3-[4-(8-Chloro-5,6-dihydro-benzo[5,6] cyclohepta[1,2-b]pyridin-11-ylidene)-piperidin-1-yl]-2-methyl-indan-1-one (C23) was prepared from desloratadine (0.15 g, 0.5 mmol) and 3-bromo-2-methyl-indan-1-one (0.11 g, 0.5 mmol) as described in General Procedure 2. The residue of the reaction mixture was cleaned up with DCM/methanol (95:5) and a reddish oil was produced containing a mixture of two diastereomers that were not separated (0.10 g, 0.22 mmol, 46%). IR ν_{max} (NaCl plate) 1712 (C=O) cm^{-1}. δ_H ($CDCl_3$) 1.34 (3H, d, J=7.5, CH_3), 2.26-2.60 (7H, m, CH_2), 2.71-2.89 (4H, m, CH_2, $CHCH_3$), 3.34-3.44 (2H, m, CH_2), 4.14 (1H, s, br, CH_2NCH), 7.05-7.16 (4H, m, ArCH), 7.14-7.45 (2H, m, ArCH), 7.65 (1H, t, J=7.9, ArCH), 7.73 (2H, dd, J=7.5, ArCH), 8.39 (1H, m, ArCH). δ_C ($CDCl_3$) 16.3, 16.4 (CH_3), 31.4, 31.5, 31.8, 31.9 (4C, CH_2), 41.6, 41.8 ($CHCH_3$), 49.0, 49.1, 52.1 (2C, NCH_2), 71.4 (NCH), 122.0 (ArCH), 123.3 (ArCH), 125.9, 126.0 (ArCH), 126.4 (ArCH), 128.7 (ArCH), 128.9, 129.0 (ArCH), 130.7, 130.8 (ArCH), 132.7 (C), 133.4 (C), 134.8, 134.9 (ArCH), 136.5 (C), 137.2, 137.2 (ArCH), 137.8 (C), 138.7 (C), 139.5 (C), 146.5, 146.6 (Ar-NCH), 157.6 (CC=O), 194.2 (C=O). *m/z* 455.1092 (MH^+, expected: 455.1890).

3-Desloratadine-propionic acid methyl ester, 3-[4-(8-Chloro-5,6-dihydro-benzo[5,6]cyclohepta[1,2-b]pyridin-11-ylidene)-piperidin-1-yl]-propionic acid methyl ester (C24) was prepared from desloratadine (0.31 g, 1 mmol) and 3-bromopropionic acid methyl ester (0.088 mg, 1 mmol) as described in General Procedure 2. A reddish oil was obtained (0.35 g, 0.88 mmol) after cleaning up the reaction mixture by flash chromatography with DCM/methanol (92.5:7.5) 88%. IR ν_{max} (NaCl plate) 1738 (C=O) cm^{-1}. H-H and C-H COSY were used for assignment of the NMR shifts. δ_H ($CDCl_3$) 2.15 (2H, m, $NCH_2CH_2C=O$), 2.36 (3H, m, CH_2) 2.49 (3H, m, CH_2), 2.69-2.90 (6H, m, CH_2), 3.38 (2H, m, CH_2), 3.66 (3H, s, OCH_3), 7.07 (1H, m, ArCH), 7.14 (3H, m, ArCH), 7.4 (1H, m, ArCH), 8.9 (1H, m, ArNCH). δ_C ($CDCl_3$) 30.6 30.8 (2C, CH_2), 31.4, 31.7 (2C, CH_2), 32.1 ($NCH_2CH_2C=O$), 51.5 (OCH_3), 53.2 ($NCH_2CH_2C=O$), 54.4 54.5 (2C, $CCCH_2CH_2N$), 122.0 (ArCH), 125.9 (ArCH), 128.9 (ArCH), 130.7 (ArCH), 132.6 (C), 132.7 (C), 133.3 (C), 137.1 (ArCH), 137.8 (C), 138.5 (C), 139.4 (C), 146.5 (Ar-NCH), 157.5 ($NCC=C$), 172.9 (C=O). *m/z* 397.1693 (MH^+, expected: 397.1683).

3-(Indan-2-ylamino)-propionic acid methyl ester (C25) was prepared from 2-aminoindane hydrochloride (0.17 g, 1 mmol) and 3-bromopropionic acid methyl ester (0.16 g, 2 mmol) as described in General Procedure 2 (in this reaction, 2 equivalents of

the ester were used to improve yield). The solvent of the reaction mixture was evaporated and the residue was cleaned up with DCM/methanol, (97.5:2.5). A brown oil was obtained (0.11g, 0.51mmol) 51 %. IRν_{max} (NaCl plate) 1736 (C=O) cm^{-1}. δ_H ($CDCl_3$) 1.62 (1H, s, br, NH), 2.56 (2H, t, J=6.5, $NHCH_2\underline{C}H_2C{=}OOCH_3$), 2.78 (2H, dd, J_{gem}=15.6, J_{vic}=6.5, $CC\underline{H}_2CHNH$), 2.97 (2H, t, J=6.5, $NHC\underline{H}_2CH_2C{=}OOCH_3$), 3.19 (2H, dd, J_{gem}=15.6, J_{vic}=7.0, $CC\underline{H}_2CHNH$), 3.66 (1H, m, $CCH_2C\underline{H}NH$), 7.14-7.17 (2H, m, Ar$\underline{H}$), 7.19-7.23 (2H, m, Ar$\underline{H}$). δ_C ($CDCl_3$) 34.7 ($\underline{C}H_2COOCH_3$), 40.0 (2C, $NHCH\underline{C}H_2$), 43.3 ($\underline{C}H_2CH_2COOCH_3$), 51.5 ($CH_3$), 59.5 (NH$\underline{C}$H), 124.6 (2C, $\underline{C}HCHCCH_2CNH$), 126.3 (2C, $CH\underline{C}HCCH_2CNH$), 141.6 (2C, $CH\underline{C}CH_2CNH$), 173.1 ($\underline{C}$=O). *m/z* 220.1345 (MH^+, expected: 220.1338).

3-Etilevodopa-indan-1-one, 3-(3,4-Dihydroxy-phenyl)-2-(3-oxo-indan-1-ylamino)-propionic acid ethyl ester (C42) was prepared from L-dopa ethyl ester (**B16**) (0.26 g, 1 mmol) and 3-bromoindan-1-one (0.21 g, 1 mmol) as described in General Procedure 2. The product was cleaned up with DCM/methanol (97.5:2.5) and a brow paste was obtained with a yield of 45% (0.16 g, 0.45 mmol). According to the NMR integration, two diastereomers were obtained with a proportion of 34/66. IR ν_{max} (NaCl plate) 1707 cm^{-1}. Assignment of the NMR shifts to protons and carbons was confirmed by C-H and H-H COSY. δ_H ($CDCl_3$) 1.19* (3H, t, J=7.2, $C\underline{H}_3$), 1.25 (3H, t, J=7.2, $C\underline{H}_3$), 2.33-2.43* (1H, m, $NHCH\underline{C}H_2C{=}O$), 2.76-2.92* (3H, m, $C\underline{H}_2CHCOOC_2H_5$, $NHCH\underline{C}H_2C{=}O$), 3.55-3.61* (1H, m, $CH_2C\underline{H}COOC_2H_5$), 4.07-4.12*, 4.19-4.25 (2H, m, $C\underline{H}_2CH_3$), 4.33-4.39* (1H, m, $NHC\underline{H}CH_2C{=}O$), 6.52-6.54 (1H, dd, J= 1.8, 7.6, $CH_2CC\underline{H}CHCOH$), 6.59-6.61* (1H, dd, J= 1.8, 8.2, $CH_2CC\underline{H}CHCOH$), 6.65 (1H, m, $CH_2CCHC\underline{H}COH$), 6.71* (1H, m, $CH_2CCHC\underline{H}COH$) 6.73-6.78* (1H, d, J=8.2, $CH_2CC\underline{H}COH$), 7.39-7.42* (1H, m, $C\underline{H}CHCHCC{=}O$), 7.53-7.57* (1H, m, $C\underline{H}CHCHCHCC{=}O$), 7.58-7.62* (1H, m, $C\underline{H}CHCC{=}O$), 7.69-7.72* (1H, m, $C\underline{H}CC{=}O$). δ_C ($CDCl_3$) 14.1*,14.2 ($\underline{C}H_3$), 39.1, 39.2* ($\underline{C}H_2CHCOOC_2H_5$), 44.5, 45.1* ($NHCH\underline{C}H_2C{=}O$), 54.6, 55.4* ($NH\underline{C}HCH_2C{=}O$), 61.2*, 61.3 ($\underline{C}H_2CH_3$), 61.4, 61.4* ($\underline{C}HCOOC_2H_5$), 115.2* ($CH_2C\underline{C}HCOH$), 116.1,116.2* ($CH_2CCH\underline{C}HCOH$), 121.2, 121.3* ($CH_2CCH\underline{C}HCOH$), 123.4*, 123.5 ($\underline{C}HCC{=}O$), 125.7, 126.3* ($\underline{C}HCHCHCHCC{=}O$), 128.8*, 128.9 ($\underline{C}HCHCHCC{=}O$), 129.1 ($CH_2\underline{C}CHCOH$),135.0*, 135.3 ($\underline{C}HCHCC{=}O$), 136.6 ($\underline{C}CHCHCHCHCC{=}O$), 143.0 ($CH_2\underline{C}CHCHCOH$), 144.0 ($CH_2CCH\underline{C}OH$), 155.1, 155.2* ($\underline{C}C{=}O$), 174.7,175.0* ($\underline{C}OOC_2H_5$), 205.0 ($CH_2\underline{C}{=}O$) *m/z* 356.1497 ($MH^+$, expected: 356.1498).

3-Dopamine-indan-1-one, 3-[2-(3,4-Dihydroxy-phenyl)-ethylamino]-indan-1-one (C43) was prepared from dopamine hydrochloride (0.19 g, 1 mmol) and 3-bromoindan-1-one (0.21 g, 1 mmol), according to General Procedure 2 with the exception that the mixture was stirred for 3 days. The product was cleaned up by flash chromatography

with DCM/methanol (95:5) affording a yellowish solid (50 mg, 0.18 mmol) (18%) with a melting point of 198-200°C (with degradation). IR ν_{max} (KBr) 3311 (OH) 1693 (C=O) cm^{-1}. H-H and C-H COSY were used for assignment of the NMR shifts. δ_H ($CDCl_3$/($(CD_3)_2SO$)) 2.84 (2H, m, $NHCH_2C\underline{H_2}$), 2.85 (1H, dd, J_{gem}=19.0, J_{vic}=3.0, $CHC\underline{H_2}C$=O), 3.04 (1H, dd, J_{gem}=19.0, J_{vic}=7.5, $CHC\underline{H_2}C$=O), 3.15 (2H, m, $NHC\underline{H_2}CH_2$), 5.03 (1H, d, J=6.0, NC<u>H</u>), 6.48 (1H, dd, J=2.0, 8.0, $CH_2CC\underline{H}CHCOH$), 6.65 (1H, d, J=2.0, $CH_2CCHC\underline{H}COH$), 6.68 (1H, d, J=8.0, $CH_2CC\underline{H}COH$), 7.59 (1H, t, J=7.52, C<u>H</u>CHCC=O), 7.72-7.77 (2H, m, C<u>H</u>C<u>H</u>CHCHCC=O), 8.05 (1H, d, J=8.0, C<u>H</u>CC=O). 8.46 (1H, s, CO<u>H</u>), 8.48 (1H, s, CO<u>H</u>). δ_C ($CDCl_3$/($(CD_3)_2SO$)) 30.0 ($\underline{C}H_2CH_2NH$), 38.0 ($CH\underline{C}H_2C$=O), 45.9 ($CH_2\underline{C}H_2NH$), 53.5 ($\underline{C}HCH_2C$=O), 114.3 ($HOC\underline{C}HCCH_2CH_2NH$), 114.6 ($\underline{C}HCHCCH_2CH_2NH$), 118.0 ($CH\underline{C}HCCH_2CH_2NH$), 121.8 ($\underline{C}HCC$=O), 125.9 ($\underline{C}CH_2CH_2NH$), 126.2 ($\underline{C}HCHCHCHCC$=O), 129.0 ($\underline{C}HCHCHCC$=O), 133.6 ($\underline{C}HCHCC$=O), 136.1 ($\underline{C}CHCHCHCHCC$=O), 142.7 ($HO\underline{C}CHCHC$), 143.8 ($HO\underline{C}CHC$), 146.2 ($\underline{C}C$=O), 198.7 ($\underline{C}$=O). *m/z* 284.1295 ($MH^+$, expected: 284.1287).

General Procedure 3: <u>Methylation of secondary amines</u>

Tertiary methylamines were prepared from the corresponding secondary amines by methylation with methyl iodate. The reactions were carried out in acetone under reflux. Five equivalents of potassium carbonate and five equivalents of methyl iodide were added to each equivalent of the original compound.

3-(Indan-1-yl-methyl-amino)-indan-1-one (C04) was prepared form 3-(Indan-1-ylamino)-indan-1-one (**C01**) (0.26 g, 1 mmol) as described in General Procedure 3. A mixture of two diastereomers was obtained in the form of a brown oil after purification by flash chromatography with hexane/ethyl acetate (8:2) (75 mg, 0.27 mmol) 27%. The presence of two diastereomers was confirmed by NMR with a ratio of 65:35 and were not separated. After a few days, colourless crystals formed. MP 58-60°C. IR ν_{max} (KBr) 1713 cm^{-1}. δ_H ($CDCl_3$) 1.92* (3H, s, $C\underline{H_3}$), 2.09* (2H, m, $NHCH\underline{C}H_2CH_2$), 2.61* (1H, dd, J_{gem}=19.0, J_{vic}=7.0, $CHC\underline{H_2}C$=O), 2.68 (1H, dd, J_{gem}=18.6, J_{vic}=7.0, $CHC\underline{H_2}C$=O), 2.75-2.91* (2H, m, $CHC\underline{H_2}C$=O, $NHCHCH_2C\underline{H_2}$), 2.95-3.04* (1H, m, $NHCHCH_2C\underline{H_2}$), 4.37 (1H, t, J=7.5 NHC<u>H</u>CH_2CH_2), 4.59 (1H, dd, J=3.5, 7.0, C<u>H</u>CH_2C=O), 4.65* (1H, t, J=7.5 NHC<u>H</u>CH_2CH_2), 4.80* (1H, dd, J=4.0, 7.0, C<u>H</u>CH_2C=O), 7.23-7.28* (3H, m, Ar<u>H</u>), 7.43-7.52* (2H, m, Ar<u>H</u>), 7.60-7.72* (1H, m, Ar<u>H</u>), 7.75-7.79* (1H, m, Ar<u>H</u>) 7.84-7.88* (1H, m, Ar<u>H</u>). δ_C ($CDCl_3$) 26.8*, 27.3 ($\underline{C}H_2CH_2CH$), 28.2* ($\underline{C}H_3$), 30.4, 30.6* ($CH_2\underline{C}H_2CH$), 34.3 ($\underline{C}H_3$), 38.1*, 39.0 ($CH\underline{C}H_2C$=O), 58.2, 61.8* ($\underline{C}H$), 67.1, 70.0* ($\underline{C}H$), 122.8, 122.9* (Ar<u>C</u>H), 124.5, 124.6, (2C, Ar<u>C</u>H), 124.7*, 124.8* (2C, Ar<u>C</u>H), 126.3* (Ar<u>C</u>H), 126.4 (Ar<u>C</u>H), 126.4, (Ar<u>C</u>H), 126.5*, (Ar<u>C</u>H), 127.4* (Ar<u>C</u>H), 128.4, 128.5* (Ar<u>C</u>H), 134.7*,

134.8 (ArCH), 137.0*, 137.1 (C), 143.1*, 143.2 (C), 143.8*, 144.0 (C), 156.2*, 156.4 (CC=O), 204.7* (C=O). *m/z* 278.1553 (MH^+, expected: 278.1545).

3-(Indan-2-yl-methyl-amino)-indan-1-one (C05) was prepared form 3-(Indan-2-ylamino)-indan-1-one (**C02**) (0.13 mg, 0.5 mmol) as described in General Procedure 3. The reaction mixture was cleaned up in silica with hexane/ethyl acetate (8:2). After evaporation of the significant fractions, the product was recrystalised with hexane and white needle crystals (55 mg, 0.2 mmol) were obtained with 40% yield. MP 102-104°C. IR ν_{max} (KBr) 1702 cm^{-1}. δ_H ($CDCl_3$) 2.09 (CH_3), 2.62 (1H, dd, J_{gem}=18.6, J_{vic}=7.0, $CHCH_2C=O$), 2.81 (1H, dd, J_{gem}=18.6, J_{vic}=3.5, $CHCH_2C=O$), 3.10 (4H, m, $CHCH_2Ar$), 3.57 (1H, m, $NHCHCH_2Ar$), 4.82 (1H, dd, J=3.5, 7.0, $NHCHCH_2C=O$), 7.17-7.25 (4H, m, ArH), 7.47 (1H, t, J=7.0, ArH), 7.69 (1H, t, J=7.0, ArH), 7.78 (2H, t, J=8.3, ArH). δ_C ($CDCl_3$) 33.2 (CH_3), 36.0 ($CHCH_2C=O$), 37.8, 38.1 (2C $CHCH_2Ar$), 59.8 ($NHCHCH_2C=O$), 65.2 ($NHCHCH_2Ar$), 123.1 (CHCHCC=O), 124.4, 124.5 (2C, $NHCH_2CCHCH$), 126.4 (2C, $NHCH_2CCHCH$), 126.5 (CHCC=O), 128.5 (CHCHCHCHCC=O), 134.8 (CHCHCHCC=O), 137.3 (C), 141.3, 141.5 (2C), 204.6 (C=O). *m/z* 278.1543 (MH^+, expected: 278.1545).

General Procedure 4: preparation of aminobenzyl acetophenones and aminobenzyl acetones (liquid amines) by Michael addition in solid state

These compounds were prepared from the α,β unsaturated ketone (benzalacetophenone (chalcone) or benzalacetone) by dissolving it, with stirring, in an equimolar quantity of the amine at room temperature without solvent. Typically, after approximately 5-10 min, the solutions of benzalacetophenone solidified affording the sought products. They were not further purified. Benzalacetone afforded mixtures of products when primary amines were used.

4-Phenyl-4-piperidin-1-yl-butan-2-one (C28) was prepared in solid state from benzalacetone (0.30 g, 2 mmol) and piperidine (0.17 g, 2 mmol) according to General Procedure 4. It was obtained as yellowish oil (0.23 g, 2 mmol) that was confirmed to be pure by NMR 100%. IR ν_{max} (Na Cl plate) 1715 (C=O), δ_H ($CDCl_3$) 1.28 (2H, qi, J=5.2, $NCH_2CH_2CH_2$), 1.48 (4H, m, $2xNCH_2CH_2CH_2$), 2.06 (3H, s, CH_3), 2.24 (2H, m, NCH_2CH_2), 2.36 (2H, m, NCH_2CH_2), 2.75 (1H, dd, J_{gem}=15.5, J_{vic}=7.5, $NCHCH_2C=O$), 3.04 (1H, dd, J_{gem}=15.5, J_{vic}=7.5, $NCHCH_2C=O$), 3.99 (1H, t, J=7.52, $NCHCH_2C=O$), 7.18-7.21 (3H, m, CCHCHCHCHCH), 7.26 (2H, m, CCHCH). δ_C ($CDCl_3$) 24.2 ($NCH_2CH_2CH_2$), 26.0 (2C NCH_2CH_2), 30.0 (CH_3), 46.6, 50.6 (2C NCH_2CH_2), 65.5 (NCH), 126.9 (1C, ArCH), 127.6, (2C, ArCH), 128.0, (2C, ArCH), 138.3, (CCHNH), 207.0 (C=O). *m/z* 232.1703 (MH^+, expected: 232.1701).

1,3-Diphenyl-3-piperidin-1-yl-propan-1-one (C35) was prepared from piperidine (0.17 g, 2 mmol) and chalcone (0.42 g, 2 mmol) as described in General Procedure 4. A white solid was obtained (0.59 g, 2 mmol) 100% and δ_H ($CDCl_3$) is in agreement with literature[178]. MP 84-86 °C, IR ν_{max} (KBr) 1664 (C=O). δ_H ($CDCl_3$) 1.36 (2H, m, $NCH_2CH_2C\underline{H}_2$), 1.52 (4H, m, $2xNCH_2C\underline{H}_2CH_2$), 2.35 (2H, m, $NC\underline{H}_2CH_2$), 2.47 (2H, m, $NC\underline{H}_2CH_2$), 3.41 (1H, dd, J_{gem}=16.0, J_{vic}=7.5, $NCHC\underline{H}_2C=O$), 3.62 (1H, dd, J_{gem}=16.0, J_{vic}=6.5, $NCHC\underline{H}_2C=O$), 4.24 (1H, dd, J=6.5, 7.5, $NC\underline{H}CH_2C=O$), 7.25 (1H, m, Ar$C\underline{H}$), 7.31 (4H, m, Ar$C\underline{H}$), 7.45 (2H, m, Ar$C\underline{H}$), 7.55 (1H, m, Ar$C\underline{H}$), 7.93 (2H, d, J=7.5 Ar$C\underline{H}$). δ_C ($CDCl_3$) 24.5 ($NCH_2CH_2C\underline{H}_2$), 26.3 (2C, $NCH_2C\underline{H}_2$), 42.1 ($NCHC\underline{H}_2$), 51.3 (2C, $NCH_2C\underline{H}_2$), 65.8 ($N\underline{C}H$), 127.0 (Ar$\underline{C}H$), 127.9 128.0 (4C, Ar$\underline{C}H$), 128.3, 128.4 (4C, Ar$\underline{C}H$), 132.7 (Ar$\underline{C}H$) 137.4 ($\underline{C}CHNH$), 139.9 ($\underline{C}C=O$) 198.7 ($\underline{C}=O$). *m/z* 294.1861 (MH^+, expected: 294.1858).

3-Propylamino-1,3-diphenyl-propan-1-one (C36) was prepared from n-proylamine (0.12 g, 2 mmol) and chalcone (0.42 g, 2 mmol) as described before in General Procedure 4. The procedure yielded 100% of the product (0.54 g, 2 mmol) as a white solid. MP 40-42 °C. IR ν_{max} (KBr) 1678 (C=O) cm^{-1}. δ_H ($CDCl_3$) 0.92 (3H, m, $C\underline{H}_3$), 1.48 (2H, m, $C\underline{H}_2CH_3$), 2.37 (1H, br, s, N$\underline{H}$), 2.45 (2H, m, $NHC\underline{H}_2CH_2$), 3.30 (1H, dd, J_{gem}=17.6, J_{vic}=4.5, $NHCHC\underline{H}_2C=O$), 3.38 (1H, dd, J_{gem}=17.6, J_{vic}=8.0, $NHCHC\underline{H}_2C=O$), 4.32 (1H, dd, J=4.5, 8.0, $NHC\underline{H}CH_2C=O$), 7.28 (1H, m, Ar$C\underline{H}$), 7.36 (2H, m, Ar$C\underline{H}$), 7.44 (4H, m, Ar$C\underline{H}$), 7.54 (1H, m, Ar$C\underline{H}$), 7.93 (2H, d, J=7.5, Ar$C\underline{H}$). δ_C ($CDCl_3$) 11.7 ($\underline{C}H_3$), 23.1 ($\underline{C}H_2CH_3$), 47.2 ($CH\underline{C}H_2C=O$), 49.6 ($\underline{C}H_2CH_2CH_3$), 58.9 ($NH\underline{C}HCH_2$), 127.2 (3C, Ar$\underline{C}H$), 128.0 (2C, Ar$\underline{C}H$), 128.5 (2C, Ar$\underline{C}H$), 133.1 (Ar$\underline{C}H$), 136.8 ($\underline{C}CHNH$) 143.6 ($\underline{C}C=O$)) 198.9 ($\underline{C}=O$). *m/z* 268.1710 (MH^+, expected: 268.1701).

3-Hexylamino-1,3-diphenyl-propan-1-one (C37) was prepared from n-hexylamine (0.20 g, 2 mmol) and chalcone (0.42 g, 2 mmol), as described in General Procedure 4. The procedure yielded 100% of the sought product as a white solid (0.62g, 2 mmol). MP and δ_H ($CDCl_3$) are in agreement with literature[178]. MP 48-50 °C, IR ν_{max} (KBr) 3309 (NH), 1678 (C=O), 2920, 2853 (aliphatic C-H) cm^{-1}. δ_H ($CDCl_3$) 0.89 (3H, t, J=7.0, $C\underline{H}_3$), 1.28 (6H, m, $CH_2C\underline{H}_2C\underline{H}_2C\underline{H}_2CH_3$), 1.46 (2H, qi, J=7.0, $NHCH_2C\underline{H}_2CH_2$), 1.86 (1H, br, s, *NH*), 2.46 (2H, m, $NHC\underline{H}_2CH_2$), 3.30 (1H, dd, J_{gem}=17.1, J_{vic}=4.5, $NHCHC\underline{H}_2C=O$), 3.37 (1H, dd, J_{gem}=17.1, J_{vic}=8.5, $NHCHC\underline{H}_2C=O$), 4.31 (1H, dd, J=4.5, 8.5, $NHC\underline{H}CH_2$), 7.28 (1H, m, *p*- Ar$C\underline{H}$), 7.36 (2H, m, Ar$C\underline{H}$), 7.44 (4H, m, Ar$C\underline{H}$), 7.56 (1H, m, Ar$C\underline{H}$), 7.93 (2H, m, Ar$C\underline{H}$). δ_C ($CDCl_3$) 14.0 ($\underline{C}H_3$), 22.5 ($\underline{C}H_2CH_3$), 26.9 ($\underline{C}H_2CH_2CH_3$), 30.1 ($\underline{C}H_2CH_2CH_2CH_3$), 31.7 ($\underline{C}H_2CH_2CH_2CH_2CH_3$), 47.2 ($NH\underline{C}H_2CH_2CH_2CH_2CH_2CH_3$), 47.6 ($CH\underline{C}H_2C=O$), 59.0 ($NH\underline{C}HCH_2$), 127.1 (3C, Ar$\underline{C}H$), 128.0 (2C, Ar$\underline{C}H$), 128.4 (2C,

ArCH), 128.5 (2C, ArCH), 133.1 ArCH), 136.8 (CCHNH) 143.6 (CC=O) 198.9 (C=O). *m/z* 310.2183 (MH^+, expected: 310.2171).

3-Cyclopentylamino-1,3-diphenyl-propan-1-one (C38). The solventless reaction of cyclopenthylamine (0.17 g, 2 mmol) with chalcone (0.42 g, 2 mmol), as described in General Procedure 4, produced **C38** as a white solid (0.59 g, 2 mmol) contaminated with the reagents (27% remained unreacted) as confirmed by NMR. 73%. MP 60-62 °C, IR ν_{max} (KBr) 1603 (C=O) 2857, 2954 (aliphatic C-H) cm^{-1}. δ_H ($CDCl_3$) 1.46 (1H, s, br, NH), 1.46-1.89 (8H, m, NHCHCH_2CH_2CH_2CH_2), 2.89 (1H, t, J=6.8 NHCHCH_2CH_2), 3.28 (1H, dd, J_{gem}=17.0, J_{vic}=4.5, NHCHCH_2C=O), 3.36 (1H, dd, J_{gem}=17.0, J_{vic}=8.0, NHCHCH_2C=O), 4.39 (1H, dd, J=4.5, 8.0, NHCHCH$_2$C=O), 7.29-7.66 (8H, m, ArH), 8.04 (2H, m, ArH). δ_C ($CDCl_3$) 23.5, 23.7 (NHCHCH_2CH_2CH_2), 32.0, 33.8, (NHCHCH_2CH_2CH_2CH_2), 47.3, (NHCHCH_2C=O), 57.0 (NHCHCH_2C=O), 57.2 (NHCHCH_2CH_2), 127.1 (ArCH), 127.2 (2C, ArCH), 127.9 (2C, ArCH), 128.4 (2C, ArCH), 128.4 (2C, ArCH), 133.0 (ArCH), 136.8 (CCHNH) 143.6 (CC=O) 198.8 (C=O). *m/z* 294.1857 (MH^+, expected: 294.1858).

3-(Indan-1-ylamino)-1,3-diphenyl-propan-1-one (C39) was produced from *R*-1-aminoindane (0.13 g 1 mmol) and chalcone (0.21 g, 1 mmol) as described in General Procedure 4. A mixture of two diastereomers was obtained with a ratio of 25:75 as confirmed by NMR and contained unreacted chalcone. 1.6-1.8 (1H, m, NHCHCH_2CH_2), 1.84 (1H, s, br, NH), 1.95-2.04* (1H, m, NHCHCH_2CH_2), 2.12-2.20 (1H, m, NHCHCH_2CH_2), 2.44-2.51* (1H, m, NHCHCH_2CH_2), 2.65-2.7 (1H, m, NHCHCH_2CH_2), 2.7-2.8* (1H, m, NHCHCH_2CH_2), 2.8-2.90 (1H, m, NHCHCH_2CH_2), 2.98-3.05 (1H, m, NHCHCH_2CH_2), 3.29-3.45* (2H, m, NHCHCH_2C=O), 4.01*, 4.13 (1H, t, J=6.8, NHCHCH_2CH_2) 4.59* (1H, dd, J_{gem}=8.8, J_{vic}=3.4 NHCHCH$_2$C=O), 4.68 (1H, dd, J_{gem}=8.2, J_{vic}=4.8 NHCHCH$_2$C=O), 7.19-7.91 (12H, m, ArH), 7.96 (2H, d, J= 7.5, ArH). δ_C ($CDCl_3$) (for the dominant diastereomer) 30.1 (CH_2), 37.3 (CH_2), 47.6 (CH_2), 58.6 (CH), 60.4 (CH), 122.4 (ArCH), 122.6 (ArCH), 124.3 (ArCH), 125.3 (ArCH), 125.5 (ArCH), 125.6 (2C, ArCH), 126.1 (ArCH), 126.6 (2C, ArCH), 126.7 (2C, ArCH), 127.1 (ArCH), 130.9 (ArCH), 135.1 (C), 141.4 (C), 141.8 (C), 144.1 (CC=O), 197.04 (C=O). *m/z* 342.1848 (MH^+, expected: 342.1858).

3-Phenethylamino-1,3-diphenyl-propan-1-one (C40) resulted from the reaction between chalcone (0.42 g, 2 mmol), and phenylethylamine (0.24 g, 2 mmol) as described in General Procedure 4. A white solid was produced (0.66 g, 2 mmol) that was proved to be pure by NMR, 100%. MP 74-76 °C, IR ν_{max} (KBr) 1671 (C=O) cm^{-1}. δ_H ($CDCl_3$) 2.69-2.82 (4H PhCH_2CH_2NH), 3.29 (1H, dd, J_{gem}=17.1, J_{vic}=5.0,

NHCHC$\underline{H}_2$C=O), 3.36 (1H, dd, J_{gem}=17.1, J_{vic}=8.0, NHCHC$\underline{H}_2$C=O), 4.36 (1H, dd, J=4.5, 8.0, NHC$\underline{H}$CH$_2$), 7.15-7.30 (6H, m, Ar$\underline{H}$), 7.37 (4H, m, Ar$\underline{H}$), 7.45 (2H, m, Ar$\underline{H}$), 7.58 (1H, m, Ar$\underline{H}$), 7.92 (2H, m, Ar$\underline{H}$). δ_C ($CDCl_3$) 36.4 (Ph-$\underline{C}H_2$), 47.2 (CH$\underline{C}$H$_2$C=O), 48.9 ($\underline{C}$H$_2$NH), 58.9 (NH$\underline{C}$HCH$_2$), 126.0 (Ar$\underline{C}$H), 127.2 (2C, Ar$\underline{C}$H), 127.3 (Ar$\underline{C}$H), 128.0 (2C, Ar$\underline{C}$H), 128.3 (2C, Ar$\underline{C}$H), 128.5 (4C, Ar$\underline{C}$H),128.6 (2C, Ar$\underline{C}$H), 132.8 Ar$\underline{C}$H), 136.4 ($\underline{C}$CHNH), 139.6 ($\underline{C}$CH$_2$CH$_2$), 142.9 ($\underline{C}$C=O), 198.4 ($\underline{C}$=O). *m/z* 330.1874 (MH^+, expected: 330.1858).

3-Desloratadine-1,3-diphenyl-propan-1-one hydrobromide salt, 3-[4-(8-Chloro-6,11-dihydro-5H-benzo[5,6]cyclohepta[1,2-b]pyridin-11-yl)-piperidin-1-yl]-1,3-diphenyl-propan-1-one hydrobromide salt (C41) was prepared from desloratadine (0.31 g, 1 mmol) and benzalacetophenone. The amine was dissolved in water/acetonitrile/ethanol (95:2.5:2.5) with benzalacetophenone. 1 mmol of hexadecyltrimethylamonnium bromide was added and the reaction mixture was stirred at room temperature overnight. The precipitated product was separated by filtration and dried under vacuum. A off-white solid was afforded (0.25 g, 0.42 mmol) 42%. MP 116-118°C. IR ν_{max} (KBr) 1683 (C=O), 1664 (C=C), cm^{-1}. δ_H ($CDCl_3$) 1.66 (N$\underline{H}$), 1.98-2.03 (1H, m, CH_2), 2.16-2.48 2.02-2.29 (5H, m, C=C$\underline{C}$H$_2$ & C=CCH$_2$C$\underline{H}_2$N), 2.69-2.82 (4H, m, C=CCH$_2$C$\underline{H}_2$N & C$\underline{C}$H$_2$CH$_2$C), 3.26-3.38 (3H, m, CCH$_2$C$\underline{H}_2$C & NCHC$\underline{H}_2$C=O), 3.66 (1H, dd, J_{gem}=16.4, J_{vic}=6.8, NCHC$\underline{H}_2$C=O), 4.26 (1H, t, J=6.5, NC$\underline{H}$), 7.04-7.40 (7H, m, Ar$\underline{H}$), 7.41-7.67 (6H, m, Ar$\underline{H}$), 7.92 (2H, d, J=8.2, C$\underline{H}$CC=O). δ_C ($CDCl_3$) 31.3 (2C, Ph-$\underline{C}H_2$), 31.7 (2C, C$\underline{C}$H$_2$CH$_2$N), 44.3 (2C, CH$\underline{C}$H$_2$C=O), 44.3 (NH$\underline{C}$HCH$_2$), 51.2, 52.3 (2H, $\underline{C}$H$_2$NH), 121.9 (Ar$\underline{C}$H), 125.8 (Ar$\underline{C}$H), 127.2 (Ar$\underline{C}$H), 127.9 (2C, Ar$\underline{C}$H), 128.1 (4C, Ar$\underline{C}$H), 128.5 (2C, Ar$\underline{C}$H), 128.8 (Ar$\underline{C}$H), 128.9 (Ar$\underline{C}$H), 130.8 (Ar$\underline{C}$H), 132.3 ($\underline{C}$), 132.5 ($\underline{C}$), 132.9 (Ar$\underline{C}$H), 133.3 ($\underline{C}$), 137.2 (Ar$\underline{C}$H), 137.7 ($\underline{C}$), 139.0 ($\underline{C}$), 139.4 ($\underline{C}$), 139.8 ($\underline{C}$), 146.5 (Ar$\underline{C}$H), 157.5 ($\underline{C}$), 198.6 ($\underline{C}$=O) *m/z* 519.2206 (MH^+, expected: 519.2203).

3-Dopamine-1,3-diphenyl-propan-1-one hydrochloride salt, 3-[2-(3,4-Dihydroxy-phenyl)-ethylamino]-1,3-diphenyl-propan-1-one hydrochloride salt (C44) was prepared from dopamine hydrochloride (0.19 g, 1 mmol) and benzalacetophenone (2.29 g, 11 mmol). The amine was dissolved in DMF with a large excess of benzalacetophenone. 1 mmol of triethylamine was added and the reaction mixture was stirred at room temperature until complete consumption of the amine (followed by CE). The solvent was evaporated and the residue was redissolved in water containing 1 mmol of HCl. This solution was extracted with DCM to remove the excessive ketone, and the aqueous solution was evaporated to dryness. The residue was washed up with a mixture of hexane and methanol (50:50). A off white solid was obtained (0.25 g, 0.63 mmol) 63 %. MP 220-222 °C. IR ν_{max} (KBr) 3328 (OH), 1686 (C=O), cm^{-1}. δ_H ($(CD_3)_2SO$)

2.71 (2H, m, $NHCH_2C\underline{H}_2$), 2.87 (2H, m, $NHC\underline{H}_2CH_2$), 4.03 (1H, dd, J_{gem}=17.6, J_{vic}=9.0, $NHCHC\underline{H}_2C{=}O$), 4.11 (1H, dd, J_{gem}=17.6, J_{vic}=4.5, $NHCHC\underline{H}_2C{=}O$), 4.83 (1H, m, br, $NHC\underline{H}CH_2C{=}O$), 6.38 (1H, d, J=8.0, $CH_2CC\underline{H}CHCOH$), 6.54 (1H, s, $CH_2CC\underline{H}COH$), 6.65 (1H, d, J=7.5, $CH_2CCHC\underline{H}COH$), 7.34-7.44 (3H, m, Ar$\underline{H}$), 7.53 (2H, t, J=7.8, Ar$\underline{H}$), 7.64 (1H, m, Ar$\underline{H}$), 7.69 (2H, m, Ar$\underline{H}$), 7.92 (2H, d, J=8.0, Ar$\underline{H}$), 8.80 (1H, s, NH^+), 9.69, (1H, s, O$\underline{H}$), 9.91 (1H, s, O$\underline{H}$). δ_C ($(CD_3)_2SO$) 31.0 ($\underline{C}H_2CH_2NH$), 41.7 ($CH\underline{C}H_2C{=}O$), 46.5 ($CH_2\underline{C}H_2NH$), 57.6 ($\underline{C}HCH_2C{=}O$), 115.9 ($HOC\underline{C}HCCH_2CH_2NH$), 116.0 ($\underline{C}HCHCCH_2CH_2NH$), 119.2 ($CH\underline{C}HCCH_2CH_2NH$), 128.1 (2C, Ar$\underline{C}$H),128.6 (Ar$\underline{C}$H),128.8 (2C, Ar$\underline{C}$H),128.9 (2C, Ar$\underline{C}$H), 129.2 (2C, Ar$\underline{C}$H), 133.9 (Ar$\underline{C}$H), 135.2 ($\underline{C}CH_2CH_2NH$), 136.1 ($\underline{C}CHNH$), 144.2 ($\underline{C}C{=}O$), 145.4 (2C, $HO\underline{C}\underline{C}OH$), 195.8 ($\underline{C}{=}O$). *m/z* 362.1758 ($M(-Cl^-)$, expected: 362.1756).

General Procedure 5: <u>Manich reaction; preparation of aminomethyl acetophenones and aminobenzyl acetophenones</u>

Aminomethyl acetophenones and some aminobenzyl acetophenones were tentatively prepared through the Mannich reaction between acetophenone, paraformaldehyde or benzaldehyde and the appropriate amine. Equimolar concentrations of the ketone, the aldehyde and the amine were kept under reflux in ethanol for over two hours in the presence of one equivalent of HCl. The precipitated products were separated by filtration and washed with ethanol. Only compound **C34** was obtained with a suitable degree of purity.

3-(4-Chloro-phenylamino)-1,3-diphenyl-propan-1-one hydrochloride salt (C34) was prepared according to General Procedure 5 from acetophenone (0.24 g, 2 mmol), benzaldehyde (0.21 g, 2 mmol), and chloroaniline (0.25 g, 2 mmol). A white solid was obtained (0.55 g, 1.5 mmol) 74%. MP170-172°C. IR ν_{max} (KBr) 1666 (C=O) cm^{-1}. δ_H ($CDCl_3$) 3.43 (1H, dd, J_{gem}=16.0, J_{vic}=7.5, $C\underline{H}_2$), 3.52 (1H, dd, J_{gem}=16.0, J_{vic}=5.0, $C\underline{H}_2$), 4.96 (1H, dd, J=5.0, 7.5, $NHC\underline{H}CH_2$), 6.49 (2H, d, J=9.0, NHCC$\underline{H}$), 7.04 (2H, d, J=9.0, $C\underline{H}CClC\underline{H}$), 7.26 (1H, t, J=7.0, $NHCHCCHCHC\underline{H}$) 7.34 (2H, t, J=7.5, $NHCHCC\underline{H}$), 7.42-7.49 (4H, m, $C\underline{H}CHCC{=}O$ & $NHCHCCHC\underline{H}$), 7.59 (1H, $C\underline{H}CHCHCC{=}O$), 7.92 (2H, d, J=7.5, $CHCC{=}O$). δ_C ($CDCl_3$) 46.2 ($\underline{C}H_2$), 55.0 ($\underline{C}HCH_2$), 115.0 (2C, NHC$\underline{C}$H), 122.5 ($\underline{C}Cl$), 126.3 (2C, NHCHC$\underline{C}$H), 127.5 ($NHCHCCHCH\underline{C}H$), 128.2 (2C, $NHCHCCH\underline{C}H$), 128.8 (2C, $\underline{C}HCHCC{=}O$), 128.9 (2C, $\underline{C}HCC{=}O$), 129.0 (2C, $NHCCH\underline{C}H$), 133.5 ($\underline{C}HCHCHCC{=}O$), 136.8 ($\underline{C}NHCH$), 142.5 ($NHCH\underline{C}$), 145.6 ($\underline{C}C{=}O$), 198.1 ($\underline{C}{=}O$) *m/z* 336.1181 ($M(-Cl^-)$, expected: 336.1155).

7.2. Assay methods

7.2.1. HPLC

High performance liquid chromatography was performed using a system consisting of a Spectra System SCM1000 ultrasound, Spectra System P4000 pump and controller, Spectra System AS3000 autosampler and a Spectra System UV1000 detector controlled by Chromquest Chromatography Manager. The stationary phase was a C18 (4.6x250 mm) Waters Spherisorb 10 μm column.

Isocratic chiral analyses were performed on a system consisting of a Waters 515 pump and a Waters 486 absorbance detector controlled by Millennium Chromatography Manager. A Pirkle covalent (*R,R*) Whek-O 1 spherica silica 5 micro 100 Å column, was used.

A third HPLC system was also employed consisting of a Waters 600 pump and controller, Waters 717 autosampler and a Waters 996 photodiode array detector controlled by Millennium Chromatography Manager 32. The stationary phase was also a C18 (4.6x250 mm) Waters Spherisorb 10 μm column.

For most non chiral tests a gradient program was used, starting with eluant A (37.5% (v/v) methanol, 3‰ (v/v) phosphoric acid (>85%), BDH) and 1‰ (v/v) triethylamine (Aldrich) in distilled and deionised water) for up to 10 minutes, and then adding eluant B (3‰ (v/v) phosphoric acid and 1‰ (v/v) triethylamine in methanol) up to 80%. For compounds **C43** and **C44**, no triethylamine was present in the eluants.

The actual timetable was varied to obtain the best resolution for each test compound and corresponding degradation products.

Detection was by UV at 214, 235 or 260 nm.

7.2.2. CE methods

Capillary electrophoresis was performed in a Beckman P/ACE system 5510 equipped with a UV filter detector set at 200 or 214 nm or a diode array detector. The system also had an autosampler with cooling possibility that was modified by connection to a water circulator. The water temperature was set so that, inside the sample vials, a temperature of 37±1°C was achieved.

Fused silica capillaries were of varied lengths and internal diameter of 50 μm. Samples were loaded by pressure injection or electrokinetic injection.

For degradation tests, runs were carried out at 25°C and at constant current of 100 μA or 150 μA in the direction of the cathode. New capillaries were conditioned beforehand with 0.1M NaOH followed by deionised water for 5 min and the running buffer for 5 min. Before each run, the capillary was rinsed with the running buffer for 1 min. The running buffer was a phosphate buffer (pH 3, 100 mM) to which 100 mM of tetrabutylammonium phosphate was added. The buffer was filtered through a 0.45 μm membrane filter before use. Data acquisition was performed by the system Gold and peak areas were recorded at 200 or 214 nm for the original compound and the parent amine (when applicable).

Chiral CE methods were set for the resolution of isomers according to the description in chapter 2.

7.3. Metabolism methods

Evaluation of metabolism by P450 was carried out using Supermix™ from Gentest. Stock solutions of the test compounds of 3-6mg/ml in methanol or ACN were used.

1.5 ml of 100 mM phosphate buffer of pH=7.4 was used (sol. I) to dissolve 30mg glucose-6-phosphate, 30 mg of $NADP^+$ and 20 mg of $MgCl_2.6H_2O$ (sol. II).

Sol III consisted in 5mM of Na_2Citrate tribasic in water. glucose-6-phosphate dehydrogenase (G-6-PDH) was dissolved in sol. III to obtain 40 U/ml (sol. IV) and was stored at -20° until use.

To ependorfs containing 220 μl of sol. I, 12.5 μl of sol. II and 2.5 μl of sol. IV, 2.5 or 5 μl of each test solution were added. The solutions were warmed to 37°C and then, 10 μl of the enzyme system was added. Control samples without added enzyme were prepared in the same way to account for non-enzymatic degradation.

The solutions were kept at 37°C for the stated amount of time (1 or 2 hours) and then were quenched with acetonitrile (150 μl). They were then centrifuged at 10,000 g for three minutes and the supernatant analysed by CE and/or HPLC using the methods described in the relevant sections of this chapter.

In order to validate the Supermix™ assay, a test was carried out with testosterone. The results were compared with the ones obtained with CYP3A4, which is the enzyme responsible for the metabolism of testosterone to 6-β-hydroxytestosterone (6-HT).

The test was carried out for P450 and CYP3A4, as described previously for P450 using 0.05 μmol of testosterone (delivered in 5 μl of ACN). A control sample containing no protein was also tested. Solutions were incubated for ten minutes at 37°C after what

they were quenched with 125 μl of ACN. They were then centrifuged at 10,000 g for three minutes.

The supernatants were then injected on to a HPLC system. The gradient program started with 35% of water and 65% of methanol for 6 minutes after what, the percentage of methanol, was increased to 75% in 4 minutes and maintained for 3 minutes. Finally the percentage of methanol was further increased to 80% in three minutes and maintained for 2 minutes. The flow rate was 1.5 ml/min. Detection was made at 254 nm. Retention times were 4.8 minutes for 6-HT and 12.1 min for testosterone.

The concentrations in the different samples were calculated from calibration curves in the ranges of 0.01 to 0.04 mM for 6-HT and 0.0125 to 0.2 mM for testosterone. Correlation coefficients were higher that 0.9999 in both cases.

7.4. Kinetic studies in vitro

7.4.1. Aqueous buffer hydrolysis

For the preparation of the buffers used in the kinetic studies, two stock solutions were used. Solution A was 0.05 M of citric acid monohydrate (99% ACS, Aldrich) and 0.02 M of boric acid (crist. M&B) in distilled and deionised water. Solution B was 0.1 M in tripotassium-orthophosphate (BDH) in distilled and deionised water. The two solutions were mixed and diluted in the necessary proportions to achieve the target ionic strengths and pHs. A 744 pH meter from Metrohm was used for accurate determination of the pHs. For ionic strengths above 0.6, sodium chloride was added.

Stock solutions of the different compounds were prepared in acetonitrile or other solvent if necessary. Aliquots of the stock solutions (10-100 μl) were diluted in the buffers (1-3 ml) at the test temperature to achieve the target concentration.

Samples were inserted in the autosampler of the capillary electrophoresis or of the HPLC machines that were also pre-warmed at the test temperature. They were then analysed using one of the previously described CE or HPLC methods.

Decay in the concentration of the test compounds and/or increase in the concentration of the free amines were followed by the decrease/increase in the corresponding peak areas as detected at an adequate wavelength at time intervals.

7.4.2. Plasma and blood hydrolysis

Citrated human blood was obtained from volunteers from the School of Pharmacy, Trinity College Dublin. Plasma was separated by centrifugation (3000 r.p.m. for 10 minutes).

The stock solutions (10-100 μl) were diluted in blood or plasma (1-3 ml) to achieve the target concentration.

Aliquots from plasma samples were taken at time intervals and quenched with a 5% (v/v) perchloric acid solution or a 4% (v/v) pherchloric acid and 1 g/l EDTA solution (1.5/1 v/v). These quenched samples were centrifuged for 3 minutes at 10000 r.p.m. and the supernatant analysed by CE or HPLC.

Blood samples were treated in the same way after separation of the plasma or were quenched directly with a larger volume (3/1 v/v), centrifuged and analysed.

7.5. Studies in Vivo

7.5.1. Absorption in the everted rat gut

Studies on the absorption in the everted rat gut were conducted according to the method described by Lane[203].

Male Sprague Dawley albino rats were starved for 24 hours prior to the experiment. The rats were anaesthetised and then an abdominal incision was made through which the small intestines were removed from the body cavity. They were than cannulated with a glass L-shaped cannula with an internal diameter of 2 mm and connected to an infusion pump. The 33.3 cm of the intestine required for the test were measured and another cannula was introduced at that distance from the previous one.

The intestine was perfused with an isotonic phosphate buffer (pH=7.4) at 37°C until clean. A syringe was then filled with the solution to be tested and attached to the pump. The solution was advanced through the cannula to the proximal cannulation point and at the start of the experiment (t_0) the pump was switched to a flow rate of 0.2 ml/min.

Samples were subsequently taken every ten minutes for a period of two hours and were weighed to check the flow rate and determine any variations in the volume collected.

The test compounds were assayed by CE or HPLC according to the non chiral methods described in 7.2.2.

1 ml of 14C-PEG 4000 (Amersham, United Kingdom) was diluted to 100 ml and the diluted solution was added at a ratio of 1 ml diluted radiolabelled marker to every 24 ml of perfusion solution in order to test alterations in the permeability of the intestine membrane. The samples from the absorption experiment were assayed for ^{14}C-PEG-4000 by pipetting 0.1 ml of the sample into a scintillation vial with 10 ml of Ultima Gold as a liquid scintillation cocktail. They were then sampled on a Liquid Scintillation Counter, TriCarb 4000 (Packard). The number of disintegrations per minute (DPM) of each sample was then compared with the DPM of the perfusate. The ratio was used as an indication of the fraction of ^{14}C-PEG-4000 absorbed or of the concentration factor.

7.5.2. Intravenous injection to rats and detection in blood and brain

A solution of the test compound was prepared in saline/ethanol/DMSO/1,2-propylenglykol (Riedel-de Haen 99.5%) (1:0.5:0.1:0.4) for administration in the form of a bolus injection.

Injections of 0.1 ml or 0.2 ml were administered to anaesthetised animals and blood samples were collected at time intervals. Blood samples were centrifuged (3 min 10 r.p.m) for separation of plasma and these plasma samples were deproteinised with 1.5 times its volume of a quenching solution (4% perchloric acid, 1 g/l of EDTA sodium salt).

A saline solution was administered to the rats by heart puncture, in order to remove the blood in the brain and sacrifice the animals. The brains were removed and macerated with an equivalent mass of quenching solution and the supernatant was separated by centrifuging. These samples were analysed by CE for the test compound and for the corresponding free amine.

7.6. pKa determination

The same stock solutions described in section 7.4.1 were used to prepare solutions for pK_a determination. Stocks A and B were combined to obtain solutions with an ionic strength of 0.05 and pHs in the range of 2.3 to 11.5.

Sample solutions were prepared by dilution from stock solutions (ACN) in pH=3 buffer (for unstable samples) or in water. DMSO were added as EOF marker.

A 20 cm fused silica capillary was used and kept at 20°C. The capillary was rinsed with a HCl 0.01 mM solution for 10 minute prior to the initiation of the tests, followed by water

(5 minutes) and the more acidic running buffer (5 minutes). Mobilities were then determined in this buffer. Before proceeding for determination of mobilities with the buffer immediately following in the pH scale, the capillary was rinsed with it for a five-minute period.

Each sample was tested three consecutive times at each pH and the average mobilities were calculated based on the retention time of the sample and of the EOF. The injection method was by pressure for 3 seconds and a voltage of 15 KV was applied. Each run lasted for the period necessary for elution of the EOF marker. Batches of not more than six samples were tested with the same running buffer and a sample of 3-aminophenol was included in each batch for detection of any methodological errors. PHs were measured before and after each batch test and the average pHs were taken for the calculations.

Each batch was tested three times. The results for mobilities of each sample afforded by the three tests were fitted by non-linear regression to a sigmoidal curve using GraphPad Prism[196]. The pK_as were obtained as a parameter of this curve.

ANNEX 1 NOTES ON CAPILLARY ELECTROPHORESIS

Capillary electrophoresis (CE) is often mentioned as a recent technique but in fact, electrophoretic separations in narrow diameter tubes were reported in literature as early as 1950. Nevertheless, the first experiments that were actually referred to as Capillary Zone Electrophoresis (CZE) started only in the sixties. A 1-3 mm i.d. quartz capillary was used and, due to its large volume, it had to be rotated along the longitudinal axis in order to achieve stabilisation. The next important steps occurred in the early seventies with the reduction of the diameter of the capillaries to the range 0.2-0.5 mm and the development of fused silica capillaries[326]. At this time the expressions for the calculation of peak dispersion due to Joule heating and for the electroosmotic flow (EOF) were also derived.

The most important breakthrough in the development of capillary electrophoresis occurred in 1981 when Jorgenson and Lukacs[327] used a 75 μm capillary with a field of 30 kV/m. The next important achievement was the introduction of micellar electrokinetic capillary chromatography (MECC or MEKC) by Terabe and co-workers in 1984[328] which made possible the separation of neutral compounds.

Since then, a long path has been walked and CE, in its several different modes, has been used for multiple purposes. The technique is very versatile and its use is possible in virtually any kind of compounds. When compared to its closest analytical technique, HPLC, CE is often referred to as having a better separating efficiency; being more environmental friendly; allowing the use of extremes of pH; requiring less quantity of sample material and less sample preparation; simplifying the interfaces for MS detection and enabling chiral separations at low cost.

Pharmaceutical science applications of CE can be found in many of the fields required for drug discovery and evaluation[329], like reliability of synthesis; impurity profile from synthesis or degradation; determination of dosage form content or uniformity; dissolution and disintegration tests; metabolite evaluation; bioavailability and biotransformation studies; determination of ionisation constants (pK_a), partition coefficients[330,331,332,333] , molecular weight, isoelectric point, binding constants[334,335] and complexation constants[336,337].

The method is particularly simple when applied to small ionisable compounds, when there is no need for using gels or surfactants.

Most chiral drugs display different pharmacological, pharmacokinetic, and toxicological properties for each stereoisomer. This fact has influenced the requirements of the regulatory authorities towards chiral compounds and, as a consequence, they have to be evaluated independently and it has to be possible to determine their stereochemical composition and/or purity[338]. Before the advent of CE, chiral separations were achieved mostly by chromatography, the first analytical scale chromatographic chiral separation being reported in 1966[339]. With this technique, the resolution of stereoisomers can be achieved both by the indirect or the direct mode. The indirect mode consists in derivatising the enantiomers with a chiral reagent to produce a pair of diastereomers that can then be analysed by HPLC or GC. The direct mode involves the use of chiral columns or chiral selectors in the mobile phase.

The first direct CE enantiomer separation was reported in 1985[340]. Separation of enantiomers by CE may also be achieved by off-line derivatisation of the compounds or by using coated capillaries (capillary electrochromatography-CEC) but the most common and simplest method consists in the addition of an optically pure chiral selector to the running buffer.

Chiral separations are simple to achieve by CE, at low cost, using the direct mode by adding a chiral modifier to the running buffer[341]. Many applications have emerged for enantioselective determination of drugs, namely in the fields of pharmacokinetics, metabolism, clinical and forensic toxicology[342].

Due to its simplicity, versatility and affordable cost, CE was the technique chosen for most of the determinations in the present work.

A1.1 Some basic concepts of capillary electrophoresis

Electroosmosis was first observed in 1877 by Helmholtz[343]. It consists of the movement of the liquid close to the capillary walls as a consequence of the ionisation of the inner surface

of the capillary. This happens because the silanol groups of the silica can be negatively charged above a certain pH leaving an excess of positive charges in the solution.

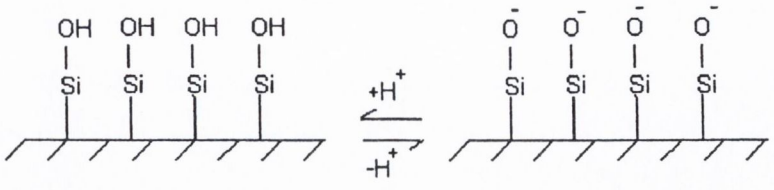


Figure A1.1: Ionisation of silanol groups in silica capillaries

When the silanol groups are ionised, a layer of positive ions from the buffer adsorbs to the wall creating a potential gradient in the interface called the zeta potential. The movement of the remaining ions results in a bulk flow of liquid to the cathode.

For this reason, cations, anions and neutral compounds move in the same direction in most systems. The migration velocity of each species is therefore dependent on its own electrophoretic mobility and on the electroosmotic flow (EOF).

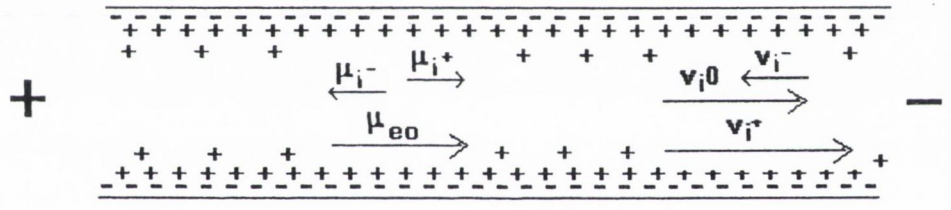


Figure A1.2: Mobility and velocity of species in the presence of EOF

The electroosmotic flow is proportional to the applied field (E) and to the coefficient of the electroosmotic flow (μ_{eo}):

$$EOF = \mu_{eo} E \qquad \textbf{(A.1)}$$

The coefficient of the electroosmotic flow depends on the dielectric constant of the buffer (ε) the zeta potential (ξ), and the viscosity of the medium (η):

$$\mu_{eo} = \frac{\varepsilon \xi}{4\pi\eta} \qquad \textbf{(A.2)}$$

The net velocity for each species is also proportional to the applied field and to the sum of the coefficient of the electroosmotic flow and the ionic mobility (bearing in mind the direction of the mobility):

$V_{i+}=(\mu_{eo}+\mu_{i+})E$ for cations **(A.3)**

$V_{i-}=(\mu_{eo}+\mu_{i-})E$ for anions **(A.4)**

$V_{i0}=\mu_{eo}E$ for neutral species **(A.5)**

Even though anions have the tendency to migrate to the anode, they are dragged to the cathode by the EOF. Also the neutrals, which do not possess electric mobility, migrate to the cathode although they cannot be separated as they all move at the velocity of the EOF.

A typical electrophoregram, obtained with direct polarity (movement from the anode to the cathode), has the following migration order based on the charge and the size of the species:

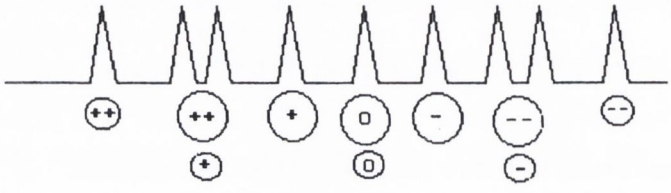


Figure 2.29: Order of elution in capillary electrophoresis

A1.2 Determination of pK_as

A1.2.1 History and recent developments

In developing new drugs, the dissociation constants have great importance as they may affect passage across membranes and other body barriers[344]. They are key parameters for understanding reaction rates, biological activity, transport and transformation[345]. During the drug development process, the knowledge of the ionisation constant may help to elucidate its structure and indicate conditions under which the compounds can be isolated at maximum yield[170]. Potentiometric titration, UV spectrophotometry, conductimetry and solubility methods are traditionally used for the determination of pK_as[170] but these methods cannot be applied on very insoluble or unstable drugs[345]. Furthermore, any impurities in the samples can greatly influence the accuracy of the determination.

Raman and NMR spectroscopy and thermometric titrations have been used mainly for pK_a values outside the range 2-11[170].

Reverse phase liquid chromatography can also be used for the determination of pK_as, as the degree of ionisation influences the retention time of the molecules. However, the range of stability of the columns limits the application of the method. Some stationary phases have been developed to broaden the range of application but the method does not always give accurate results[346, 347].

Four decades ago, some reports started to mention the use of electrophoretic methods for the determination of pK_as[348,349]. At the time, the supporting medium used was paper and

the mobilities were measured by reference to a standard substance known to be completely ionised over the pH range being considered[348]. The method was also used for the estimation of molecular weights[349].

Later, isotachophoresis (ITP) was often used for the determination of absolute mobility and pK_a[350,351,352,353,354] but the method was laborious, involved computer simulations and was limited to the range of pH of 3-11[353].

The determination of pK_as and mobilities by ITP has been referred as useful for fingerprinting unknown compounds like pollutants or additives[355].

In the last decade several pK_a determinations involving capillary zone electrophoresis (CZE) were reported[344,353,356,357,358,359,360,361,362,363,364,365,366] and the method was further developed. Many limitations of other methods are overcome by this technique, including the range of pH that can be used and the purity of the sample[344]. There is no need of parameter correction as in ITP, since the composition of the background electrolyte can be considered to be nearly constant in terms of ionic strength, pH, temperature and electric field[353]. Furthermore, simultaneous determination of the pK_as of mixed samples is possible[364] and unstable compounds can be analysed as long as the response of the detector offers a good peak[361], since the concentration of the sample does not affect mobility[356,359]. Determination of the different pK_as of molecules with more than one ionisable group is also possible[357].

The method can be used even for nearly insoluble compounds by using an organic modifier in the running buffer and extrapolating the results to zero concentration of the modifier[361]. This approach has also been used for potentiometric determinations, but in that case it is prone to errors as the plot of the pK_a against the percentage of organic solvent is not usually linear[361].

Another advantage of the CE method is that, for stable compounds, it is possible to confirm the results using the same data (when using UV detection), by plotting the areas against the pH[358,367,368]. This is a variation of the traditional spectrophotometric method of determining ionisation constants and relies on the fact that the ionised and neutral states of a molecule have different absorption coefficients at the same wavelength. Relative to the classical method, this approach has the advantages of selectivity a higher sensitivity.

As in any other method, an accurate evaluation of the pH of the running buffer is very important. Some authors have tried to simplify these determinations using an internal standard of known pK_a to calculate the pH. Nevertheless, better precision is achieved if the determination is made off line by the use of a pH meter[360].

In the recent years, the number of papers referring the use of capillary electrophoresis for pK_a determination of compounds with pharmacological interest seems to be steadily increasing[347,367,369,370,371,372,373]. Developments include the use of pressure assisted CE[370,374] and the injection through the short end of the capillary[375] which further improves the speed of the determination. Authors refer to a throughput of 20 compounds per day with this technique[370] with a possibility of determining the pK_a of one compound in less than 40 min[375]. Stacking conditions have been applied, which allowed the use of concentrations as low as 2 μM[375].

The applicability of the method to zwitterionic compounds was shown[371,347]. The best computer method for fitting experimental data was investigated[372] as well as the effect of different buffer systems[372] and different organic modifiers in the mobile phase[373] and in the sample solution[347]. Results were compared to the ones obtained by computational methods[369], and chromatographic, potentiometric or spectrophotometric techniques[347,368].

Recently, a linear method based on the plot of the reciprocal effective mobility against inverse concentration of protons, was shown to provide similar results to a potentiometric method[377].

Non aqueous CE has also been used for the determination of dissociation constants[376].

Although the simplicity of the determination of pK_as by CE is widely acknowledged, the accuracy of this method hasn't yet been proved better relative to others. Nevertheless, the increasing number of papers reporting the determination of pK_as by capillary electrophoresis demonstrates its wide acceptance as an alternative technique to the traditional methods. This, together with the fact that determination by other methods would be limited due to the instability of the compounds justifies the choice of capillary electrophoresis for the determination of the pK_as of the compounds studied in this work.

A1.2.2 Theory

The theory of the determination of pK_as by CE is based on the fact that the percentage of ionisation affects the migration velocity of the compound in a particular electrophoretic system. For example, a basic compound should move faster to the cathode as the pH of the running buffer decreases.

When fully ionised, the molecule moves faster to the electrode of opposite charge, while in its neutral state it only moves with the EOF. Intermediate mobilities are a function of dissociation equilibrium and a plot of the mobilities against the pH of the running buffer originates a sigmoidal curve where the inflexion point corresponds to the pK_a.

The effective mobility of ionic species (*Me*) inside a capillary at a determined pH, is a function of the electrophoretic velocity (v) and of the field strength (E).

$$M_e = \frac{v_e}{E} \qquad \textbf{(A.6)}$$

In its turn, the electrophoretic velocity is dependent on the distance between the injection point and the detector (L_d) and the migration time (t), while the field strength is dependent on the applied voltage (V) and the total capillary length (L_c).

$$v_e = \frac{L_d}{t} \qquad and \qquad E = \frac{V}{L_c} \qquad \textbf{(A.7)}$$

However, in CE, migration times are affected by the electroosmotic flow (EOF) and for this reason, the observed (or apparent) mobilities (M_{app}) are not exactly the same as the theoretical ones (M_e).

$$M_{app} = \frac{v_{app}}{E} = \frac{L_c L_d}{t_{app} V} \qquad \textbf{(A.8)}$$

The difference between the two corresponds to the contribution of the electroosmotic flow and can be calculated using the migration time of an uncharged substance[353,369]:

$$M_e = M_{app} - M_{EOF} = \frac{L_c L_d}{V}\left(\frac{1}{t_{app}} - \frac{1}{t_{EOF}}\right) \qquad \textbf{(A.9)}$$

On the other hand, the effective mobility varies with the pH of the running buffer accordingly to the degree of dissociation (α) of the ionic components:

$$M_e = \alpha M_a \qquad \textbf{(A.10)}$$

where M_a is the absolute electrophoretic mobility of the fully ionised species.

Using as an example the acid equilibrium,

$$AH \rightleftharpoons A^- + H^+$$

It follows that:

$$\frac{[A^-]}{[HA]} = \frac{\alpha}{1-\alpha} = \frac{M_e}{M_a - M_e} \qquad \textbf{(A.11)}$$

On the other hand, the thermodynamic dissociation constant for this equilibrium is given by:

$$K_a^{th} = \frac{(H^+)[A^-]}{[HA]}\gamma_{A^-} \qquad \textbf{(A.13)}$$

with the assumption that, for the neutral species *HA*, the activity coefficient is =1. Then,

$$pK_a^{th} = -\log\gamma_{A^-} + pH - \log\frac{[A^-]}{[HA]} \qquad \textbf{(A.14)}$$

The activity coefficient can be estimated from the Debye-Hückel's theory at 25°C:

$$-\log\gamma = \frac{0.5085 \cdot Z^2 \cdot \sqrt{I}}{1 + 0.3281 \cdot a \cdot \sqrt{I}} \qquad \textbf{(A.15)}$$

where *I* is the ionic strength of the medium, *a* is the hydrated diameter of the ion (usually assumed to be 5 Å) and *Z* its valence.

Including it in equation **A.14**, gives the Hendersson-Haselbelch equation (**A.16**), which correlates pK_a^{th} with the experimentally accessible data, *pH*, M_e and M_a.

$$pK_a^{th} = pH - \log\left(\frac{M_e}{M_a - M_e}\right) + \frac{0.5085 \cdot Z^2 \cdot \sqrt{I}}{1 + 0.3281 \cdot a \cdot \sqrt{I}} \qquad \textbf{(A.16)}$$

Different authors use different approaches in the way they use this equation. Some assume that, for diluted solutions (I <0.05) the term corresponding to the activity coefficient can be neglected and equation **3.22** can be rearranged to

$$M_e = \frac{M_a}{10^{(pK_a - pH)} + 1} \qquad \textbf{(A.17)}$$

for an acid or

$$M_e = \frac{M_a}{10^{(pH - pK_a)} + 1} \qquad \textbf{(A.18)}$$

for a protonated base.

This equation represents a sigmoidal model, where M_a and pK_a can be calculated by non-linear regression[369]. However, theoretically, it is possible to calculate these two unknown parameters simply by determining the effective mobilities at two different pHs.

For higher ionic strengths, a correction may be applied to account for the activity coefficients[362,363].

One possible approach to non-linear regression is to look at equations **A.17** and **A.18** as particular case of the Boltzmann sigmoid, which has the most general mathematical form **A.19**.

$$y = \frac{a}{b + \exp\left(\frac{x+c}{d}\right)} + e \qquad \textbf{(A.19)}$$

Where *a*, *b*, *c*, *d* and *e* are constants[372]. By applying this equation to a mobility shift of a proton from a protonated base as a result in a change in pH (*dpH*), and considering *b*=1 and *dpH*=log *e*, the resultant sigmoid, after converting the exponential to base 10, is[369]:

$$M_e = \frac{M_a - M_0}{1 + 10^{(pH - pK_a^{th})}} + M_0 \qquad \textbf{(A.20)}$$

where M_0 represents the mobility of the less ionised species, which is evidently zero.

ANNEX 2 STRUCTURES OF THE COMPOUNDS PREPARED FOR THIS THESIS

This annex presents the structures of the compounds synthesised during the course of the work that lead to this thesis as a quick reference for the reader.

Compounds C## correspond to the prospective prodrugs and side products obtained during their synthesis, while compounds B## correspond to intermediate products and products of degradation.

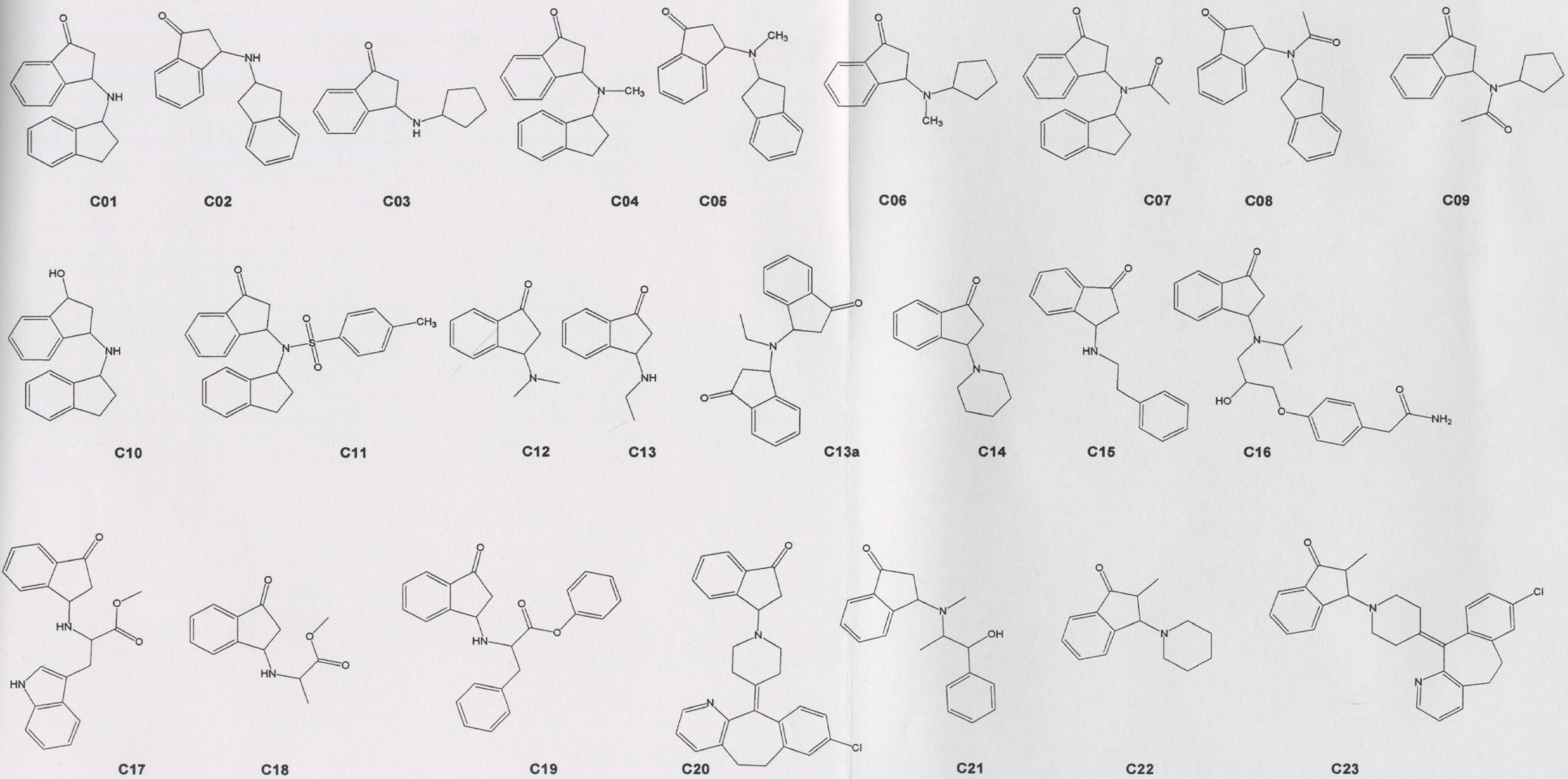


Figure A2.1: Target compounds, chapters 2 and 3

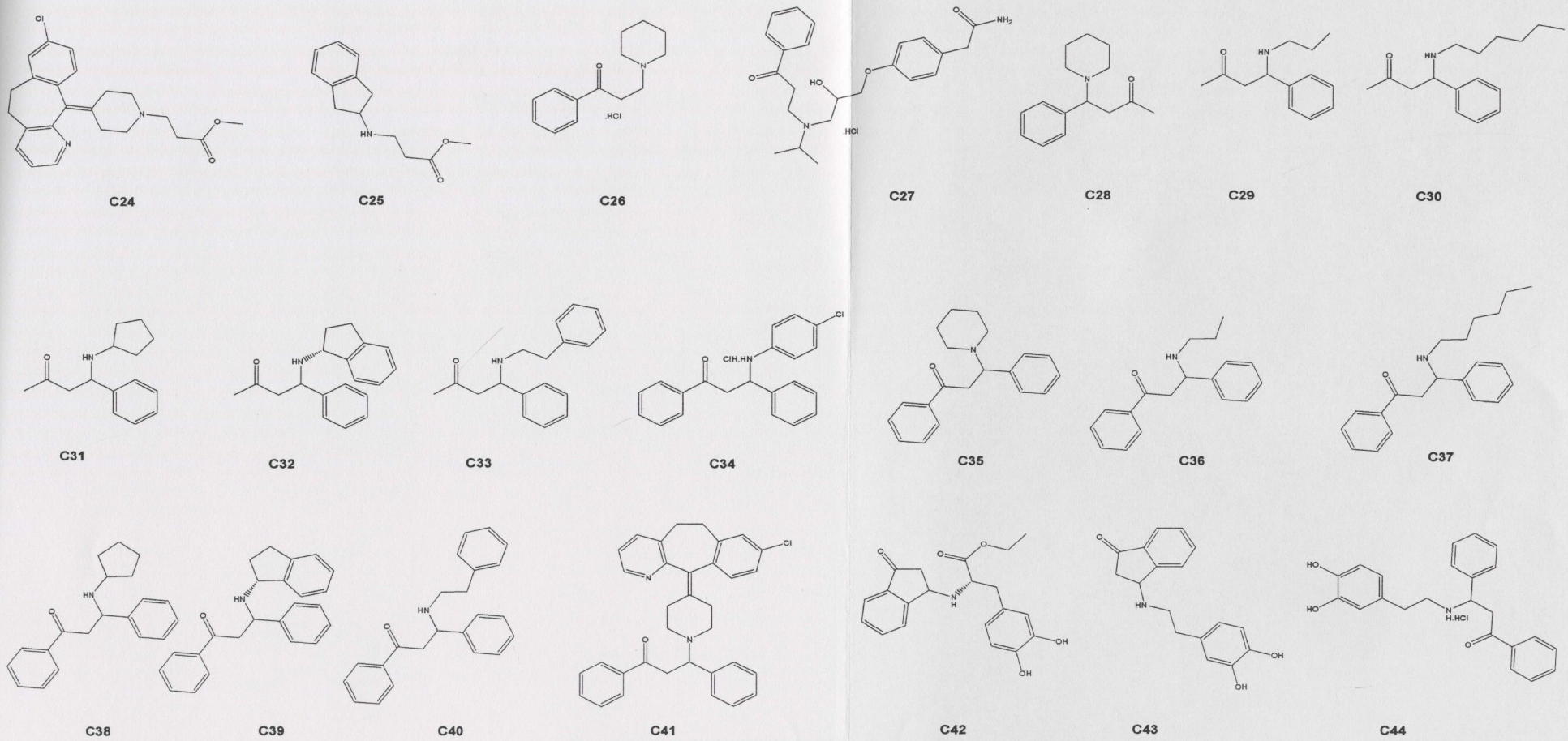


Figure A2.2 Target compounds, chapters 4 and 5

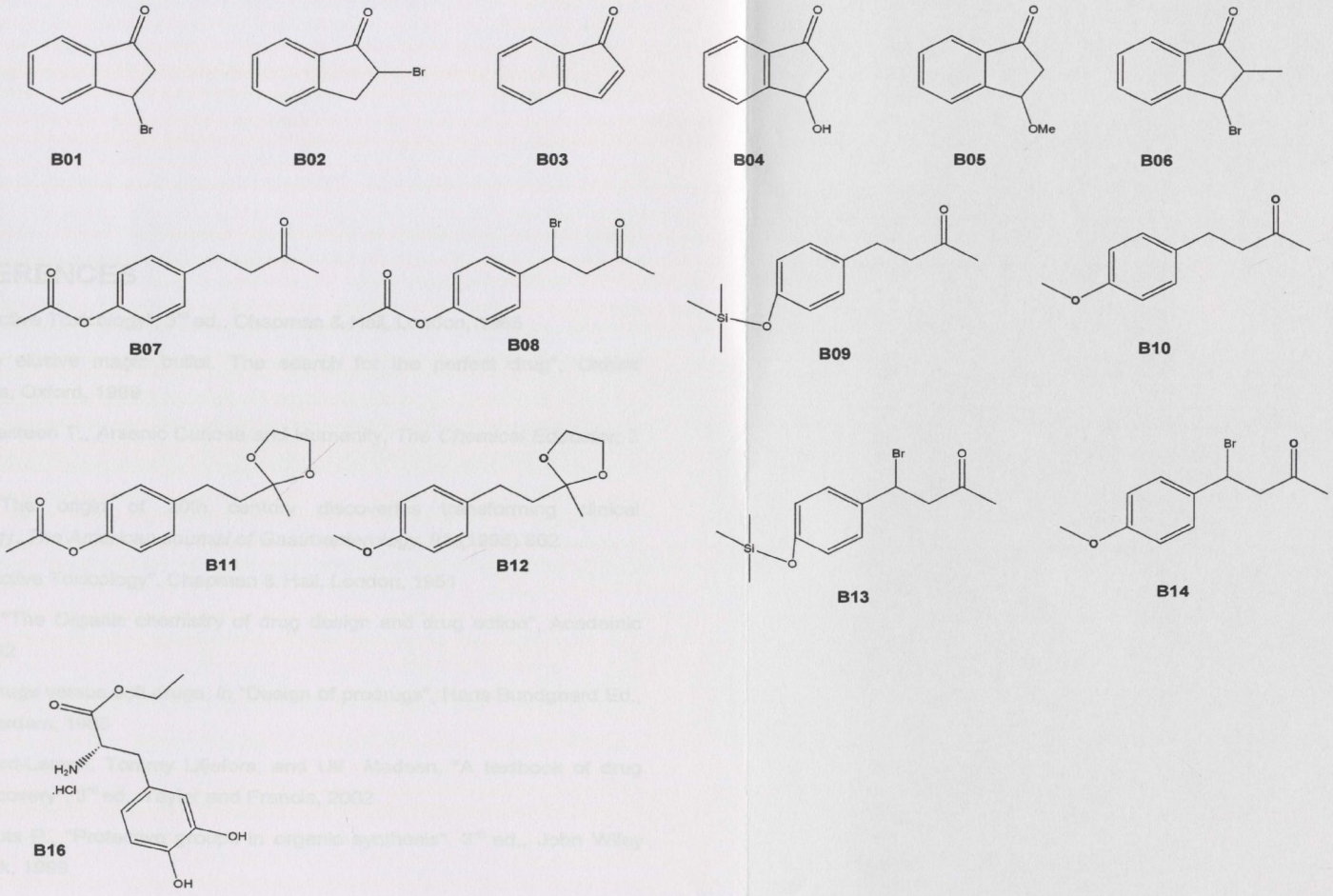


Figure A2.3: Intermediates and degradation products

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