THE STUDY OF THE POTENTIATION OF ANTICHOLINERGIC SIDE EFFECTS OF TRICYCLIC ANTIDEPRESSIVES BY FEMALE SEX STEROIDS

A Thesis submitted to

RHODES UNIVERSITY

in partial fulfilment of the requirements

for the degree of

MASTER OF SCIENCE

by

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December, 1980

ACKNOWLEDGEMENTS

It is with sincere gratitude that the following acknowledgements are made.

Professor B. Potgieter and Dr. B. J. Wilson for their unfailing advice, guidance and co-operation during research.

Mrs. P. C. Estment for the typing of this manuscript.

Mr. L. H. Purdon for his concern and co-operation.

Finally, to my wife Jane, for her interest and encouragement and for making this all possible.

INDEX

INDEX (continued)

INDEX (continued)

ABBREVIATIONS USED

cm	centimetre
°C	degrees centigrade
DMI	desmethylimipramine
DDMI	desdimethylimipramine
DNA	deoxyribonucleic acid
g	gram
g x 1000	gravity
HPLC	High Pressure Liquid Chromatography
IDB	iminodibenzyl
IM	imipramine
IMNO	imipramine N-oxide
K_A	dissociation constant
K_S	spectral dissociation constant
min	minute
ml	millilitre
mm	millimetre
mg	milligram
mmole	millimole
M	molar
mμmole	millimicromole
NADPH	Nicotinamide Adenine Dinucleotide Phosphate
nm	nanometre
%	per cent
RNA	ribonucleic acid
rpm	revolutions per minute
w/v	weight/volume
∞	infinity

<	less than
λ	wavelength
µg	microgram
µℓ	microlitre
2OHIDGB	2 hydroxyiminodibenzyl glucuronide
2OHIDB	2 hydroxyiminodibenzyl
2OHIM	2 hydroxyimipramine
2OHIMG	2 hydroxyimipramine glucuronide
2OHDMI	2 hydroxydesmethylimipramine
2OHDMIG	2 hydroxydesmethylimipramine glucuronide
2OHDDMI	2 hydroxydesdimethylimipramine
2OHDDMIG	2 hydroxydesdimethylimipramine glucuronide
BSA	bovine serum albumin

LIST OF FIGURES

LIST OF FIGURES (continued)

LIST OF FIGURES (continued)

LIST OF TABLES

ABSTRACT

It has been recorded that women respond to tricyclic antidepressives with a greater incidence of anticholinergic side effects than men do, particularly women taking an exogenous source of oestrogen.

The aim of this study was to investigate the influence that ethinyl oestradiol and Premarin ^{R} had on the metabolism of a number of tricyclic antidepressives, and also the influence they had on the binding ability of microsomes to imipramine.

Rat hepatocyctes and microsomes were used. Detection techniques used were High Pressure Liquid Chromatography and Spectrophotometry respectively.

In addition to these studies, a study of the anticholinergic activity of Nomifensine, tricyclic antidepressives and their derivatives was performed on a rat jujenum.

Results conclusively showed that ethinyl oestradiol had a marked influence on the metabolism of the tricyclic antidepressives studied. Premarin ^{R} had little, if any influence. However, both ethinyl oestradiol and Premarin ^{R} affected the binding of microsomes to imipramine, but ethinyl oestradiol had the greater effect.

The parent compound in each case exhibited a higher pA_2 value.

Results indicate that a possible explanation for the increased anticholinergic side effect is due to an inhibition of the metabolism of the tricyclic antidepressives by oestrogen.

LITERATURE REVIEW

SECTION 1

CLASSIFICATION OF MENTAL DISORDERS

Mental disorder can be divided into two main groups (Merital Scientific)[1]

Neurosis and

Psychosis

1.1 Neurosis

Neurotic people have exaggerated, normal reactions. Because of poor inter-personal relationships and due to the fact that they are incapable of handling their own personal emotional conflicts, these people regard themselves as inadequate and they consequently become anxious.

This anxiety or fear may either present as an anxiety neurosis or the patient might process it in one of the following ways:

a) He may act in an obsessive complusive fashion, in other words, his daily routine is occupied with numerous small and comparatively unimportant rituals. This rigid way of existence prevents proper development of his or her personality and if such an individual cannot execute these rituals, he develops fear, anxiety, and depression (here depression is a symptom of the neurosis).

b) The other alternative is hysteria. These individuals do not experience any anxiety, but whenever circumstances of anxiety do arise they simply dissociate themselves and escape into a world of fantasy.

1.2 Psychosis

Psychotic patients have no insight into their problems and they live in a world of their own which is dominated by hallucination and delusion.

This distortion of reality is one of the main differences between neurotic and psychotic people. As a group, neurotics are able to recognise reality but the psychotics have no insight or very little insight into their condition at all.

1.2.1 Organic Psychoses

Organic psychoses are caused by organic pathology of the brain, trauma, tumours, or diminished cerebral blood supply (as in senility and arteriosclerosis).

1.2.2 Functional Psychoses

In these cases no obvious underlying physical abnormality can be detected. Functional psychoses are divided into schizophrenia and affective psychosis.

1.2.2.1 Schizophrenia

Schizophrenia accounts for approximately one fifth of patients currently residing in mental institutions, and is still one of the least understood conditions in psychiatry. In its most typical form, it consists of a slow deterioration of the entire personality which often manifests itself at a period of adolescence. It involves a great part of mental life and expresses itself in disorder of feeling, of conduct, of thought and in an increasing withdrawal of interest from the environment. The following types of schizophrenia are recognised:

a) Simple Schizophrenia

b) Paranoid Schizophrenia

c) Heberphrinic Schizophrenia

d) Catatonic Schizophrenia

But most cases are not clear cut and considerable overlap can occur.

1.2.2.2 Affective Psychoses

Affective psychoses are those conditions in which there is alteration of mood to such a degree as to cause a serious distress or disruption of normal life.

1.2.2.2.1 MENTAL DEPRESSION

Classification

There is no satisfactory classification of the depressive disorders. There are three main types of depression without adverting to an elaborate classification[2]. The first is the reactive depression. It is by far the most common and is typified by the grief reaction. Manic depressive psychosis is the second type. The third is involutional melancholia.

REACTIVE DEPRESSION

Grief - grieving occurs in response to a loss. The loss may be real, as in the death of a spouse, or imaginative or even symbolic. The typical acute grief reaction comprises five characteristics.

1. An intense subjective sensation of mental pain accompanied by a feeling of exhaustion.
2. Preoccupation with the image of the deceased.
3. A sense of guilt concerning the relationship to the deceased.
4. An inexplicable and unwarranted hostility towards friends and relatives.

5. A loss of the usual pattern of conduct. Bereaved individuals are unable either to initiate or to arrange their daily affairs and tend to perform routine tasks in an automatic and uninterested fashion.

The natural course of the normal grief reaction extends over a period of four to twelve weeks, by which time most of these five characteristics begin to abate, and within a few more months normal activities are resumed.

There are many variations of the grief reaction.

a. Pathologic Grief

The most frequent one is prolongation of the reaction; i.e., there is no sign of resolution by the end of a three month period. Mothers who have lost young children tend to suffer for a long period of time, and the elderly who have lost a spouse of many years may never recover completely. Patients with a history of previous depressive episodes may also remain in mourning for longer periods.

b. Delayed or Postponed Grief

Often in an attempt to sustain the morale of others or to avoid the unpleasant spectacle of mourning, the patient may show little or no reaction to the loss for weeks or months. Then an individual may go into a major depression.

Other Forms of Reactive Depression

Depressed patients seldom, if ever, express feelings of sadness or despair without mentioning physical concomitants such as the ease with which they tire, their loss of appetite, reduced interest in life and love, and trouble in falling asleep or in premature awakening. Therefore, whenever these symptoms become manifest in the course of

psychosis is divided into three subtypes, manic, depressive and circular.

Manic-depressive psychosis is an illness largely confined to the middle and later adult years. Not many patients develop their first attack prior to their fiftieth year, and the peak age of onset is between fifty-five and sixty-five for both sexes. The disease is two or three times more frequent among females. It is more common in individuals of Jewish and Irish heritage and occurs more frequently among those of upper socioeconomic strata. The manic forms tend to occur at an earlier age than the depressive, but at any age episodes of depression are far more frequent than are episodes of mania.

Clinical Picture

The depressive phase may develop into a full-blown picture within a surprisingly few days. The patient is apathetic and despondent, with reduced energy for mental and physical activity. There is a lack of interest in all pleasurable activity. Overwhelmed by feelings of helplessness and indecisiveness, patients become increasingly dependent and incapable of acting on their own behalf. In despair, they express the desire for death and may become suicidal with very little warning. Combination of weakness, easy fatigue, anorexia, insomnia, and loss of libido may dominate the clinical picture and may therefore simulate several medical diseases. At its worst the illness takes the form of a depressive sleep as the patient becomes mute and indifferent to nutritional needs and neglectful even of bowel and bladder functions. The condition at this time resembles catatonia. The patient must be fed and vital functions tended until therapy brings about an improvement.

The manic phase is, in most ways, the mirror opposite of the depressed phase. Patients are hyperactive with increased appetite and sexual drive. With a minimum of sleep they awake in the morning filled with joy and expectation. They appear to possess great drive and confidence, yet lack the ability to carry out their plans. Their threshold for paranoid thinking is low, which makes them sensitive and suspicious. They tend to neglect themselves to the point of poor personal hygiene.

First attacks of either depression or mania last an average of 6 months if untreated. Modern therapy can reduce this by more than half. Attacks tend to be longer in older age groups.

INVOLUTIONAL MELANCHOLIA

This is a severe depression of psychotic proportions which occurs in the involutional phase of life. It is characterized by agitation, insomnia and a profound sence of worthlessness. Multiple physical complaints, some of which may be delusional in nature, generally accompany the condition.

There appears to have been a steady increase in the prevalence of this disorder over the last 50 years. This probably reflects the growing awareness of the condition by the lay population and the appreciation that such an entity as "middle-life depression" exists and can be treated rather than an actual increased evidence. Three times as many females as males fall victim to it. There is no known cause for this sexual distribution, but some have speculated that just as many men are depressed but that it is manifested as alcoholism in these patients. Women are more apt to develop signs of involutional melancholia between the ages of fifty-one and sixty, whereas men usually fall prey to it some 5 to 10 years later. Unlike

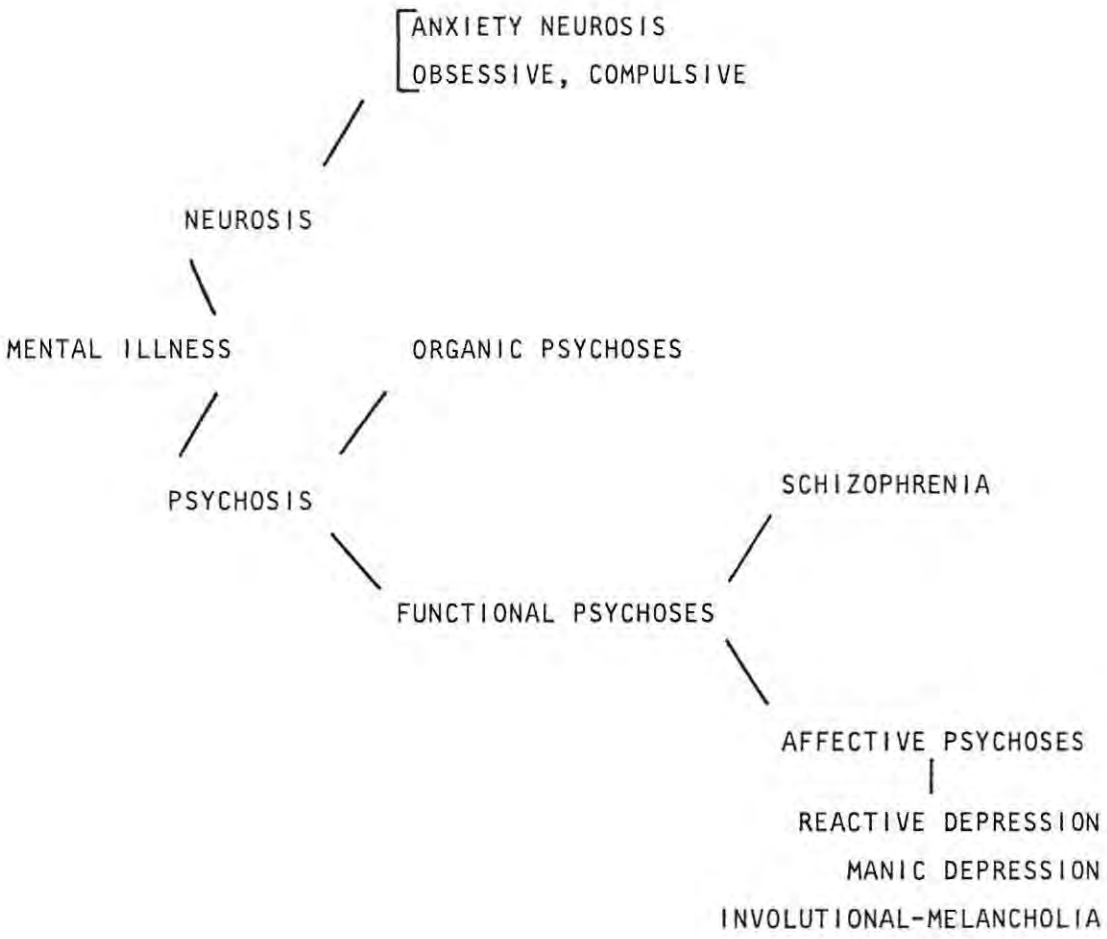
manic-depressive psychosis, this depression is more common in low socioeconomic categories.

Clinical Picture

The condition is characterized by increased irritability, insomnia, psychomotor restlessness, easy fatigue, loss of interest in sexual and gustatory pleasures, and an ever-mounting worry about health. These manifestations occur over one or more years before the clinical picture is complete. As time passes, the individual's life narrows to a single-minded concern about physical deterioration, mental decline, or both. Before long, every conversation comes to balance on the fulcrum of symptons, no matter how hard the patient may try to avoid that topic.

In contrast to the physical inactivity and mental slowness of the depressed manic-depressive patient, the principal behavioural manifestation of involutional melancholia is agitation. As a consequence these patients usually pace restlessly about the room and have great difficulty sitting still. Furthermore, they tend to be over talkative and vexed in their manner of expression.

SCHEMATIC SUMMARY



SECTION 2

CLASSIFICATION OF PSCYCHOTROPIC DRUGS

2.1 Neuroleptics: drugs of which the main therapeutic effect is on psychosis but may benefit other types of psychiatric disorders. In addition they usually produce extra pyramidal effects, such as tremor and rigidity.

a) Phenothiazines: e.g. Chlorpromazine

b) Thioxanthines : e.g. Thiothixene

c) Butyrophenones: e.g. Haloperidol

d) Diphenylbutylpiperidines: e.g. Pimozide

e) Reserpine derivatives

2.2 Anxiolytic Sedatives: substances that reduce pathological anxiety, tension and agitation, without therapeutic effect on disturbed cognitive or perceptual processes. These drugs usually raise the convulsive threshold and do not produce extrapyramidal or autonomic effects. They may produce drug dependence.

a) Propanedioles : e.g. Meprobamate

b) Benzodiazepines : e.g. Chlorodiazepoxide

c) Barbiturates : e.g. Amulobarbitone sodium

2.3 Antidepressives: drugs effective in the treatment of pathological depressive states.

a) Monoamine oxidise inhibitors: e.g. Phenylzine

b) Tricyclic and Tetracyclic compounds: e.g. Imipramine & Mianserin

c) Other: e.g. Nomifensine

2.4 Psychostimulants: drugs that increases the level of alertness and motivation.

a) Amphetamine: Dexamphetamine

b) Caffeine

SECTION 3

ANTIDEPRESSIVE DRUGS

The successful introduction of chlorpromazine into therapy in 1953 marked the beginning of the era of psycho pharmacology[3]. In the following years, scores of derivatives, mainly phenothiazines and other tricyclic compounds were synthesized in the search for new psychotropic drugs. One of these compounds, imipramine, was released to be clinically tested for its presumed antihistaminic sedative and above all neuroleptic activities. The new compound demonstrated a distinct antidepressive action as observed and formulated by the psychiatrist Kuhn.

Scores of imipramine derivatives as well as totally different structures were tested as antidepressives; only about a dozen have remained in therapeutic use.

Closely related compounds, could be divided into two groups. Imipramine (a dibenzazepine derivative) and amitriptyline (a dibenzocycloheptane derivative). The other non structural related antidepressives although not monoamine oxidase (MAO) inhibitors could be classified into monocyclic, bicyclic, tricyclic and tetracyclic antidepressives.

3.1 Tricyclic Antidepressives

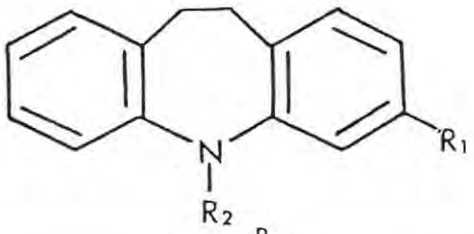
Imipramine, amitriptyline and other closely related compounds are the drugs currently used for the treatment of depression. Because of their structure (Table 1) they are often referred to as tricyclic antidepressives.

3.1.1 Pharmacology

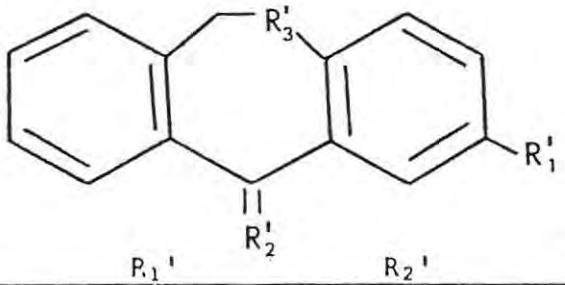
The general pharmacology of these drugs has been reviewed[4,5].

TABLE 1

IMIPRAMINE AND OTHER DIBENZAZEPINE ANTIDEPRESSIVES

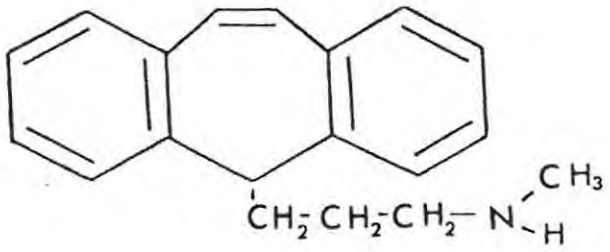


	R_1	R_2
IMIPRAMINE	H	$CH_2-CH_2-CH_2-N(CH_3)(CH_3)$
DESIPRAMINE	H	$CH_2-CH_2-CH_2-N(H)(CH_3)$
CHLORIMIPRAMINE	Cℓ	$CH_2-CH_2-CH_2-N(CH_3)(CH_3)$
TRIMIPRAMINE	H	$CH_2-CH(CH_3)-CH_2N(CH_3)(CH_3)$



	R_1'	R_2'	R_3'
AMITRIPTYLINE	H	$CH-CH_2-CH_2-N(CH_3)(CH_3)$	CH
NORTRIPTYLINE	H	$CH-CH_2-CH_2-N(H)(CH_3)$	CH
DOTHIEPIN	H	$CH-CH_2-CH_2-N(CH_3)(CH_3)$	S
DOXEPIN	H	$CH-CH_2-CH_2-N(CH_3)(CH_3)$	O

PROTRIPTYLINE



The pharmacological profile of tricyclic antidepressives is characterized by a multitude of actions which apparently do not relate with clinical antidepressive action.

3.1.1.1 Central Nervous System

Many of imipramine's effects, behavioural or otherwise, resemble those of the phenothiazines. In both animals and normal humans, sedation and decreased psychomotor activity are noted. There is no stimulatory or mood changing action except in depressed patients suggesting a peculiar specificity towards a pathologic state rather than an extension of pharmacological effects.

Effect on Sleep The tricyclic antidepressives occasionaly have been used as hypnotics because of their sedative property. Although this effect may be useful in the initial therapy of a depressed patient with sleep loss, their general use for hypnosis is not recommended.

3.1.1.2 Action on Brain Amines

The action of the tricyclic antidepressives on the metabolism of catecholamines and indoleamines in the brain has contributed significantly to the "biogenic amines hypothesis" of depression. The amines of primary interest are serotonin and noradrenaline. Tricyclic antidepressives have no significant effect on dopamine. All tricyclic antidepressives block the re-uptake of noradrenaline by adrenergic nerve terminals. The demethylated analogues are more potent in this action, whereas the methylated drugs are more potent in the blockade of serotonin re-uptake. The exact relationship of these effects to the actions of the tricyclic antidepressives in human depression is not known.

3.1.1.3 Cardiovascular System

Tricyclic antidepressives have marked effect on the cardiovascular system even in therapeutic doses[6]. There is an increased tendency for arrhythmias to develop in patients and there have been several reports of unexpected deaths due to this cause[7]. These adverse reactions may be related to the blockade of amine re-uptake by these drugs and the resultant high concentration of noradrenaline in cardiac tissue. Orthostatic hypotension is commonly observed with therapeutic doses possibly due to increase of noradrenaline in vasomotor centre. Myocardial infarction during the course of treatment has been attributed to imipramine administration. Tachycardia is a common finding. The most prominent ECG change observed following the use of imipramine consists of inversion or flattening of the T waves.

3.1.1.4 Respiration

In man, imipramine in clinical doses produces little effect on respiration. Respiratory depression has been observed following poisoning with imipramine and with amitriptyline.

3.1.1.5 Anticholinergic Effects

The tricyclic antidepressives possess peripheral[5,8,9,10,11] and central antimuscarinic activity[5,9,10,11].

The side effects commonly seen occurring in 5% - 30% of patients, include dry mouth, drowsiness or sedation and constipation. Less common side effects include extra pyramidal symptoms (usually mild and consisting of tremor, but sometimes akathisia or gait disturbance) blurred vision, postural hypotension, sweating and tachycardia. Urinary retention has been rare. Some investigators have reported instances of parasthesia, notable weight gain, excitement and leukopaenia and thrombocytopenia[5]. These and other infrequent

side effects are to be expected from tricyclic antidepressives.

MECHANISM OF ANTIMUSCARINIC ACTION

Parasympathetic Nervous System

This system consists of three outflows of preganglionic fibres from the CNS and their postganglionic connections. The regions of central origin are the midbrain, the medulla oblongata and the sacral part of the spinal cord. The parasympathetic system has its terminal ganglia very near to the organs innervated and thus is more discrete and limited in its discharge of impulses than the sympathetic nervous system. The parasympathetic system is concerned primarily with the functions of conservation and restoration of energy rather than with the expenditure of energy. It slows the heart rate, lowers the blood pressure, stimulates the gastrointestinal movements and secretions, aids absorption of nutrients, protects the retina from excessive light, and empties the urinary bladder and rectum.[12]

Acetylcholine Receptors

Acetylcholine receptors of the peripheral nervous system of vertebrates are classified into two major categories. There are nicotinic receptors, activated by nicotine and inhibited by d-tubocurarine, which are found in autonomic ganglia, skeletal muscle and in the central nervous system, and there are muscarinic receptors, activated by L(+) muscarine and inhibited by atropine, which are found in smooth muscle, cardiac muscle, ganglia, brain and glands. There are also acetylcholine receptors in mammalian brain that have been found to exhibit mixed nicotinic - muscarinic pharmacology[13].

Influence of Structure on Affinity and Intrinsic Activity

A receptor must perform two functions; firstly, it must recognise chemical features of the drug which are appropriate for forming a complex; secondly, the formation of this drug-receptor complex may lead to the initiation of a physiological change in the cell (i.e., the effect of agonists) or it may not (i.e., the effect of antagonists). In the latter case, its action is revealed in preventing the action of an agonist, or of a physiologically released transmitter. An agonist therefore will show affinity for the receptor and intrinsic activity, whereas an antagonist will only show affinity and no intrinsic activity.

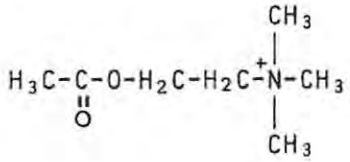
While it is highly probable that an endogenous agonist exists for each receptor, in practice the agonist may not be known and in any case the specificity of a receptor is not uniquely determined by a single ligand but rather by the range of chemical structures that interact with it. A receptor type is therefore defined by structure activity relationships.

In the case of muscarinic receptors competition between agonists and radio active labelled antagonists has shown that all the specifically bound antagonist can be displaced by a sufficient concentration of agonist[14]. In order to initiate an action it is necessary that the agonist should be able to change the character of the receptor in some way. It seems to be inescapably identified with a conformational change.

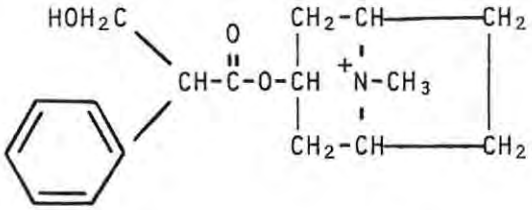
Speculating on the nature of the receptors for acetylcholine in the terminal synapse of its parasymathetic nerves studies have yielded the following information:

(1) The interaction of the ammonium groups or their equivalents with

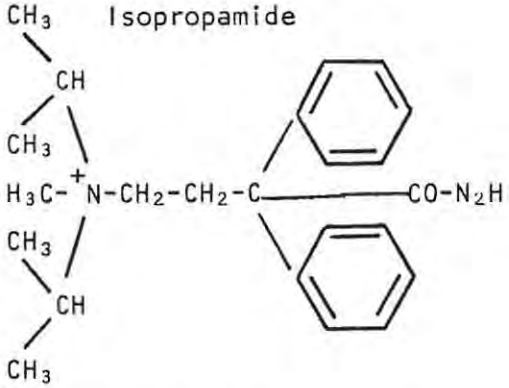
the receptors contributes to the affinity and is essential for the intrinsic activity, e.g., Acetylcholine.



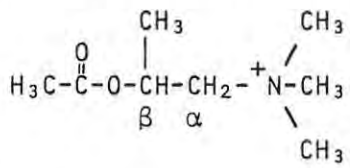
(2) Alkyl substitution on the ammonium or amino groups leads to a loss of the intrinsic activity. Also the affinity decreases, but it increases again when the alkyl or aralkyl substituents become large enough to contribute to the affinity on additional receptor parts, e.g. l. Atropine.



e.g., 2. Isopropamide

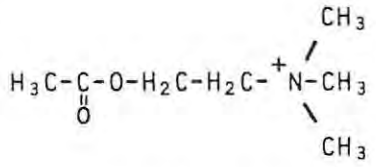


(3) Introduction of a β-methyl group in choline leads to an ℓ and d compound. The ℓ-compound is about as active as acetylcholine; the d-compound is much less active. e.g., Methacholine.

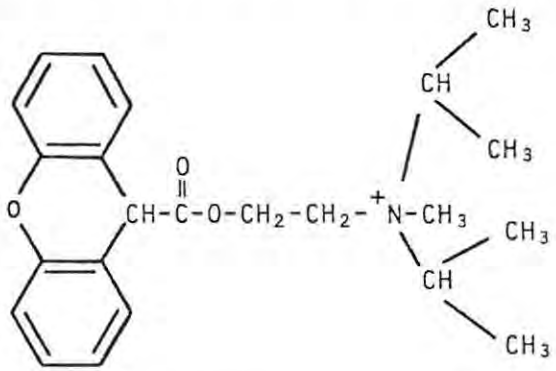


(4) The ester group in the drug molecule or its equivalent contributes to the affinity. It is not essential for the intrinsic

activity. It appears to facilitate the induction of the stimulus and therefore contributes to the intrinsic activity, e.g. Acetylcholine.



(5) Substitution of an alkyl or phenyl group and other rings on the acetic acid moiety of acetylcholine and analogous substitutions in other parasympathomimetics result in a loss in the intrinsic activity and a loss in the affinity. The loss in affinity may be compensated for, however, when binding energy is gained on additional receptor parts by large substituents, e.g. Propantheline



Practically all acetylcholinolytics have in common a positively charged group with some larger more or less planar structure at a suitable distance, and one or two phenyl or heterocylic rings. The length of the chain between the positively charged group and the ring structure is less specific; it must have a certain length, usually three to four interatomic distances.[15]

Figure 1 shows structure and specificity for mimetics and lytics.

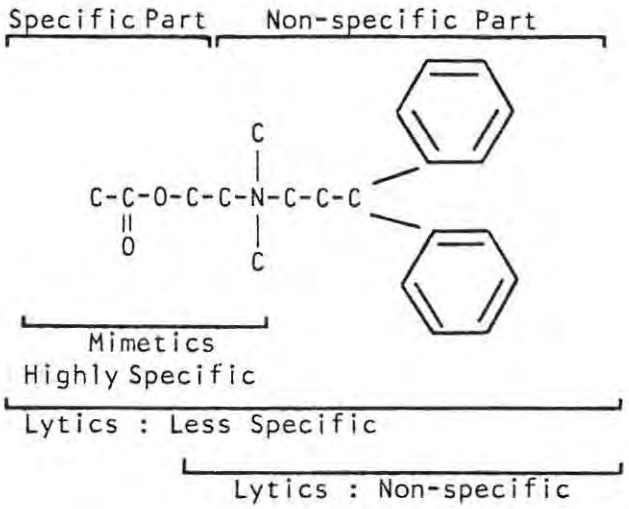


Figure 1 Mimetics and lytics, structure and specificity. Model for the relation between mimetics, lytics containing the specific structure of the mimetic and lytics without this specific structure.

Table 2 shows lytics chemically related to mimetics

Table 2

Relations in the Chemical Structure of Mimetics and Different Types of Lytics

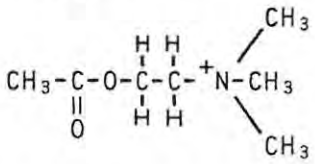
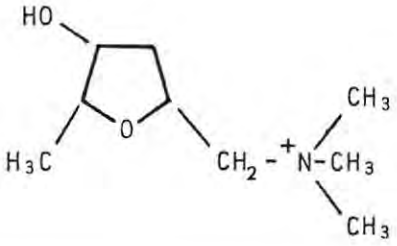
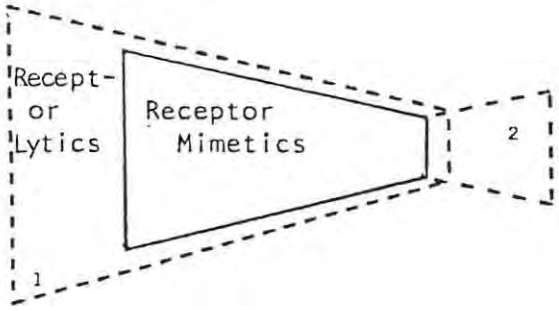
MIMETICS
Non Specific, Muscarinic and Nicotinic
e.g. Acetylcholine

Muscarine Specific
e.g. Muscarine


Table 2 cont.

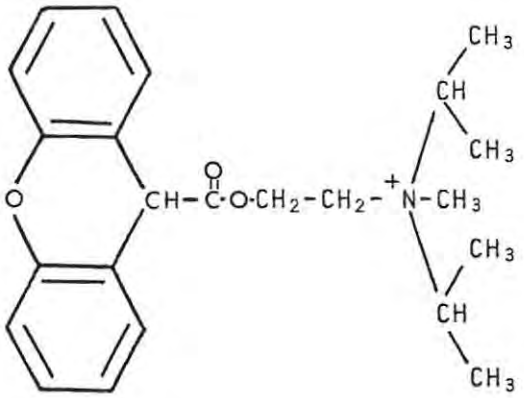
LYTICS

Very Specific for Muscarinic Receptors

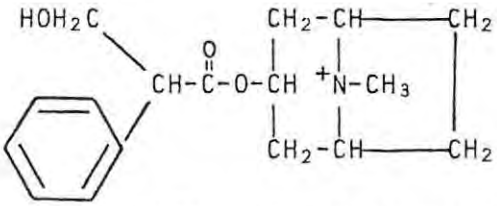


Receptor Mimetic

----- Receptor Lytic

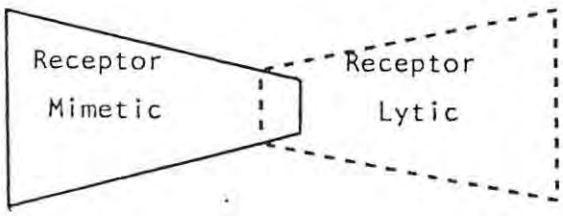


e.g. 1 Propantheline



e.g. 2 Atropine

Non-specific Multi-Potent Antagonist



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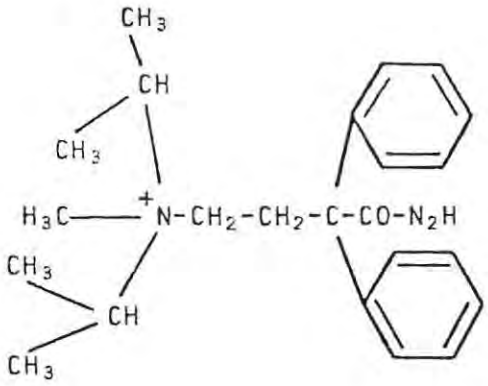
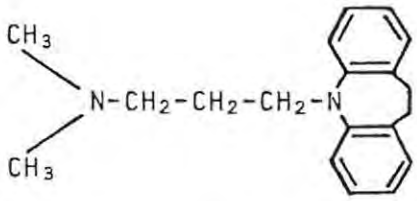


e.g. 1 Isopropamide



e.g. 2 Imipramine

From the theories put forward by Ariens[15] one can postulate the possible mechanism that tricyclic antidepressives have as anticholinergic agents. Because of the structure of tricyclic andidepressives they would be expected to act as non specific lytics, so one would expect them to show antihistaminic effects as well. In fact they do have this property, therefore they can be called multipotent antagonists.

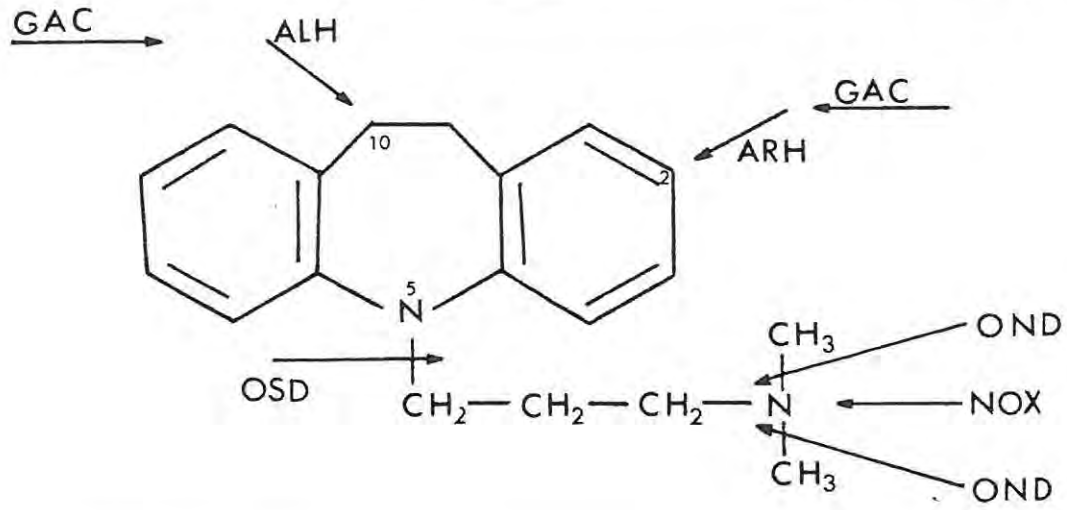
3.1.2 Metabolism

The metabolism of only a few drugs has been investigated as thoroughly as that of imipramine . There are a number of reasons for this. Imipramine is a widely used drug which, soon after its introduction, was found to form therapeutically active metabolites. Owing to several points of attack on the imipramine molecule, the drug undergoes metabolism by multiple pathways leading to some thirty metabolites. Imipramine has therefore become a model drug for the study of a complex drug metabolic situation.

Table 3 shows the metabolically vulnerable groups or bonds of the imipramine molecule and a list of metabolites known to date. Of more than twenty atom groups of the molecule, only six are known to be points of metabolic attack[3,16]. Thus all biotransformations of imipramine result in peripheral molecular changes only, leaving the tricyclic nucleus intact. All metabolites still belong to the one chemical family of benzazepine derivatives. The number of metabolites far surpasses the number of metabolically vulnerable positions. This is because two or more positions can be attacked simultaneously or in sequence leading to the formation of combination metabolites e.g. 2OH desimipramine, which is both demethylated and hydroxylated. The number of theoretically possible combinations exceeds thirty. However, certain combinations of metabolic attack exclude each other. Thus, hydroxylated N-oxides or metabolites hydroxylated both in 2 and 10 positions, have never been found. There are vast differences in the amounts of individual metabolites produced. The major imipramine metabolites in most species are desimipramine, 2 hydroxy-imipramine, 2 hydroxy-desipramine, and O-glucuronides of the latter two hydroxylated metabolites.

TABLE 3

Metabolically Vulnerable Parts of the Imipramine Molecule and Metabolites of Imipramine



METABOLITE	ABBREVIATION
2 Hydroxyimipramine	2-OH-IM
Desmethylimipramine, desipramine	DMI
2 Hydroxyimipramine glucuronide	2OH-IM-G
2 Hydroxydesmethylimipramine	2OH-DMI
2 Hydroxydesipramine glucuronide	2OH-DMI-G
Desdimethylimipramine	DDMI
Imipramine N-oxide	IMNO
Iminodibenzyl	IDB
2 Hydroxyiminodibenzyl	2OH-IDB
10 Hydroxyimipramine	10-OH-IM
10 Hydroxydesimipramine	10-OH-DMI
10 Hydroxydesdimethylimipramine	10-OH-DDMI
10 Hydroxyiminodibenzyl	10-OH-IDB
10 Hydroxyimipramine glucuronide	10-OH-IM-G
10 Hydroxydesimipramine glucuronide	10-OHDMI-G
10 Hydroxydesdimethylimipramine glucuronide	10-OHDDMI-G
10 Hydroxyiminodibenzyl glucuronide	10-OH-IDB-G
2 Hydroxydesdimethylimipramine	2-OH-DDMI
2 Hydroxydesdimethylimipramine glucuronide	2-OH-DDMI-G
2 Hydroxyiminodibenzyl glucuronide	2OH-IDB-G
Desmethylimipramine-N-glucuronide	DMI-N-G
N-Hydroxydesmethylimipramine	DMI-N-OH
N-Hydroxydesdimethylimipramine	DDMI-N-OH

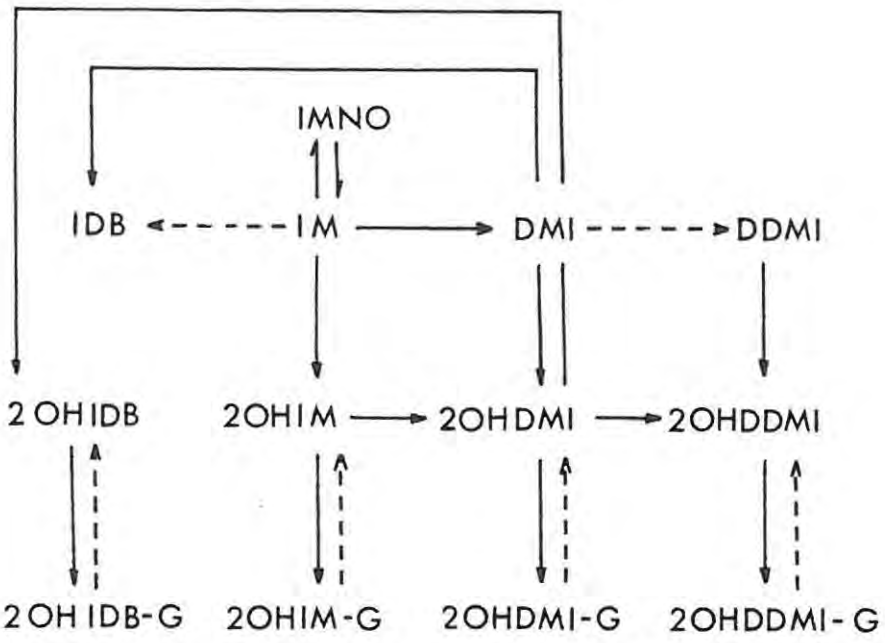
ACH, aliphatic hydroxylation; ARH aromatic hydroxylation, GAC glucuronic acid conjugation; NOX N-oxidation; OND oxidative N-demethylation; OSD oxidative side-chain dealkylation.

The predominant pathways leading to the major metabolites of imipramine are represented schematically in Table 4. Oxidative-N demethylation may occur in two steps transforming imipramine to its secondary desimipramine and primary desdimethylimipramine amine analogues. Oxidative side chain dealkylation, leading to the formation of iminodibenzyl, may be the same type of reaction which is directed towards the non basic nitrogen atom. Formation of iminobenzyl may also occur non enzymatically. Aromatic hydroxylation in position 2 is a major pathway, while aliphatic hydroxylation in position 10 is of minor importance. The phenolic and alcoholic metabolites resulting from the latter pathways serve as substrates for the important pathways of O-glucuronidation, which is often so efficient that few unconjugated hydroxylated metabolites can be detected. The pathway of N-glucuronidation can apply to secondary and primary amines only. In addition to N oxidation of imipramine, two pathways have been shown to apply to the N-oxide formed. N-oxide reduction efficiently leads back to imipramine, while N-oxide demethylation, a non oxidative process leads to dimethylimipramine. N-oxidation of the secondary and primary amine analogues results in the formation of N-hydroxylated metabolites (hydroxylamines). N-methylation of desimipramine has also been reported.

The metabolic reactions of the imipramine series are catalyzed by enzymes or enzyme systems localized in a variety of tissues and subcellular sites. A liver microsomal multienzyme system catalyzing the sequence of oxidation and glucuronide conjugation is the centre of the metabolic activity for imipramine, as for most other drugs.

TABLE 4

Scheme of the Major Pathways and Metabolites of Imipramine



The oxidative system responsible for the initial pathways of demethylation and aromatic hydroxylation is referred to as monoxygenase system; it involves the flavoprotein, NADPH cytochrome C-reductase, and cytochrome P-450 and oxygen. Imipramine forms a complex with cytochrome P-450 which results in a type I spectral change. The same has been observed with major unconjugated metabolites. An in depth study of this enzyme system will be covered in Section 5.

Formation of N-oxides from tertiary amines has been found to be independent of cytochrome P-450, but dependent on a flavoprotein amine oxidase, which is also localized in hepatic microsomes.

Considerable species differences in the metabolism of imipramine and its metabolites have been observed. Demethylation is the

predominant initial pathway in rats and guinea pigs while in pigs it is N-oxidation. Desimipramine rapidly undergoes further metabolism in mice and rabbits but accumulates in rats and humans. The sex also has an influence on metabolism of imipramine, but will be covered in Section 5.

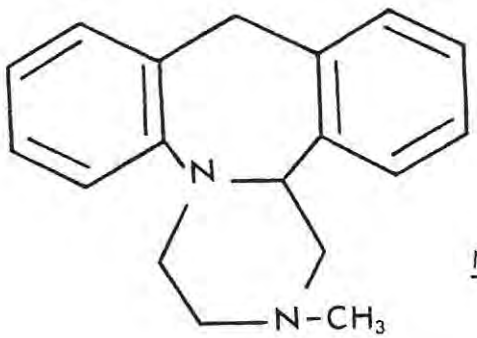
3.2. New Antidepressives

Since the introduction of imipramine by Kuhn in 1957 for the treatment of depression, a number of new antidepressive drugs have been developed by altering the tricyclic structure of the compound. However, it has been shown that drugs with different chemical structures and pharmacological and biochemical profiles also possess antidepressive properties.

The following are the more commonly used newer antidepressives for the treatment of depression.

3.3 Mianserin

Mianserin, a new antidepressive, chemically a tetracyclic piperazino-azepine (Fig. 2), was initially developed as an antihistamine and anti-serotonin compound[17]. The pharmacological profile of action of mianserin is different from that of the tricyclic antidepressives. It combines presynaptic α adrenoceptor blocking activity with antihistaminic properties, but has no central anticholinergic activity and little effect on central serotoninergic reuptake mechanisms.



Mianserin

Fig. 2

3.3.1 Pharmacology

3.3.1.1 Central Nervous System Effects

The impairment of short term memory and learning capacity caused by amitriptyline but not by mianserin despite a tendency to a greater sedative effect with mianserin, suggests that mianserin lacks central anticholinergic effects[18]. Fatigue and drowsiness have been reported during administration of both mianserin and placebo, but the incidence was significantly higher in those receiving mianserin.

Effect on sleep. Mianserin had no particular effect on healthy volunteers. However, mianserin improved sleep in depressed patients when compared with placebo.

3.3.1.2 Action on Brain Amines

In vitro or in vivo mianserin differs from the classical tricyclic antidepressives in its capacity to reduce the concentration and increase the turnover of noradrenaline in the brain.

Effect on Uptake of Noradrenaline

Mianserin does not affect noradrenaline uptake in vivo, and increases its turnover[19,20,21]. In vitro, inhibition of uptake has been reported[22]. In man therapeutic doses have no effect on peripheral noradrenaline uptake, and unlike the tricyclic antidepressives do not significantly decrease the anti-hypertensive action of the adrenergic neurone blocking drugs bellanidine[23], or guanethidine[24]. Inhibition of serotoninergic uptake mechanisms would thus not be expected in patients being treated with clinically effective doses of mianserin[22].

Effect on Serotoninergic Uptake Mechanisms

It has been reported that mianserin has essentially no effect on central

serotonin uptake *in vivo* in rats and is much less active than desimipramine or nortriptyline in inhibiting serotonin uptake by rat hypothalmic synaptosomes[22].

In patients recovering from depression, whose blood platelets showed a significantly reduced capacity to accumulate serotonin, mianserin produced an increase in serotonin transport to normal values[23], although it had no effect on serotonin uptake into platelets in healthy volunteers. Mianserin did not affect serotonin uptake by rat blood platelets *in vitro* but it did induce release of serotonin from rat brain synaptosomes[25].

3.3.1.3 Effect on Histamine Receptors

A large number of structurally different antidepressive drugs inhibit histamine - sensitive adenylate cyclase and it has been suggested that this effect may contribute to the antidepressant action of these compounds[26]. Since mianserin is also a potent inhibitor, this may contribute to its mechanism of action.

3.3.1.4 Cardiovascular Effects

Findings of studies so far suggest that mianserin is less liable to cause important adverse effects on the heart than the standard tricyclic antidepressives[27,28].

3.3.1.5 Anticholinergic Effects

The anticholinergic effects of mianserin in isolated organs[29], on intact animals[30], and in man[31], are absent or clearly much less pronounced than those of amitriptyline.

Dizziness, blurred vision, finger tremor and pronounced dry mouth occurred in a greater number of patients on amitriptyline than on mianserin[31].

The small anticholinergic effect may be attributed to a poor structure relationship with acetylcholine as discussed in Section 2.

3.3.2 Metabolism

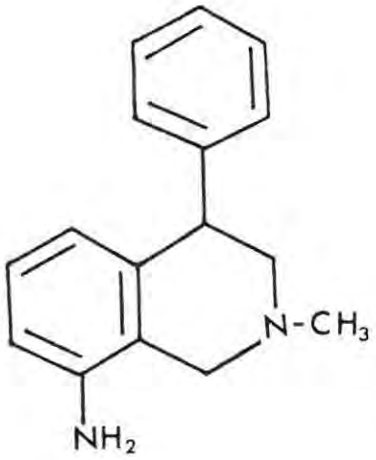
The predominant route of biotransformation of mianserin in the human is aromatic hydroxylation, N-oxidation, and N-demethylation[32]. Most of a dose is metabolized, only 4 to 7% being present in the urine as unchanged drug. There are no published data indicating whether or not the metabolites are pharmacologically active.

The mono-oxygenase system is responsible for demethylation and aromatic hydroxylation.

3.4. Nomifensine

Nomifensine is a tetrahydroisoquinoline antidepressive (Fig 3), which is chemically distinct from the tricyclic or tetracyclic antidepressives, monoamine oxidase inhibitors and other currently available agents.

It differs from the tricyclics in that it markedly inhibits the re-uptake of dopamine from presynaptic nerve terminals and has a relatively weak influence on the re-uptake of other catecholamines or serotonin.



Nomifensine

Fig. 3

3.4.1 Pharmacology

3.4.1.1 Central Nervous System Effects

Low doses reduce mobility and have slight sedative effect[33], but higher doses increase locomotor activity. The stereotype behaviour seen at higher doses may indicate presynaptic effects[34]. The 4 hydroxyphenyl (M_1) and 3 methoxy-4-hydroxyphenyl (M_2) metabolites produced stereotyped behaviour similar to that produced by nomifensine[34]. The stereotyped behaviour is due at least in part to the dopaminergic properties of the drug.

Effect on Sleep

The M_1 metabolite of nomifensine prolongs the hexobarbitone sleeping time when administered orally, whilst nomifensine itself significantly reduces the sleeping time. The M_2 and M_3 (3-hydroxy-4-methoxyphenyl derivative) metabolites have no consistent effects. Repeated doses of nomifensine 75 mg daily cause some deterioration in the ability to get to sleep, however, when compared to imipramine and placebo, some of the subjects feel more alert in the morning after receiving nomifensine[35]. On the other hand it was found that imipramine has a more beneficial effect on sleep disturbances in depressed out-patients[36].

3.4.1.2 Action on Brain Amines

Nomifensine has been shown to strongly inhibit the uptake of noradrenaline and dopamine *in vitro* into rat brain synaptosomes[37], and to inhibit dopamine uptake into human platelets *in vitro*[38]. *In vitro* nomifensine is a strong inhibitor of noradrenaline and dopamine uptake in rat brain and heart[39]. The M_1 metabolites are similar in potency to the parent compound in inhibiting dopamine uptake and

equipotent in inhibiting noradrenaline uptake. The M_2 and M_3 metabolites are less active inhibitors of the uptake of biogenic amines, but are more potent than tricyclic antidepressives in inhibiting dopamine uptake in striatal synaptosomes[40].

Nomifensine is a relatively weak inhibitor of the serotonin uptake into various animal preparations in vitro[40], exhibiting activity comparable to mianserin. Nomifensine has a moderate effect on serotonin uptake into human patelets in vitro. On the other hand, the M_1 metabolite is about 40 times more potent a compound in inhibiting serotonin uptake into rat brain synaptosomes, and has activity comparable with that of imipramine[40].

Nomifensine does not affect the release of noradrenaline and dopamine from synaptosomes[41], and does not have direct stimulating effect on dopamine and noradrenaline receptor sites.

3.4.1.3 Cardiovascular Effects

Studies on animals have shown that the effects of nomifensine are less pronounced than those of imipramine, amitriptyline or doxepin under the same experimental conditions[39].

Findings of studies suggest that nomifensine is less liable to cause important adverse effects on the heart than the tricyclic antidepressives. Nomifensine has, as yet, not been associated with serious cardiotoxicity at therapeutic dosage, and importantly on overdosage.

3.4.1.4 Anticholinergic Activity

At relatively high doses nomifensine inhibits contractions of isolated guinea pig intestine caused by cholinergic stimulation[39]. In man, nomifensine has less peripheral anticholinergic effect than amitriptyline as determined by measurement of salivary secretion[39].

3.4.2 Metabolism

Three principal metabolites formed by hydroxylation and methoxylation of the phenyl ring are found in equal proportions (about 7%) in human serum and urine[42]. Of these only the M_1 metabolite is consistently active in vitro and in vivo.

Following a single 25 mg dose, the amount of unchanged drug in serum is <5%, nomifensine being present mainly as the conjugate. The pathway can be seen in Figure 4

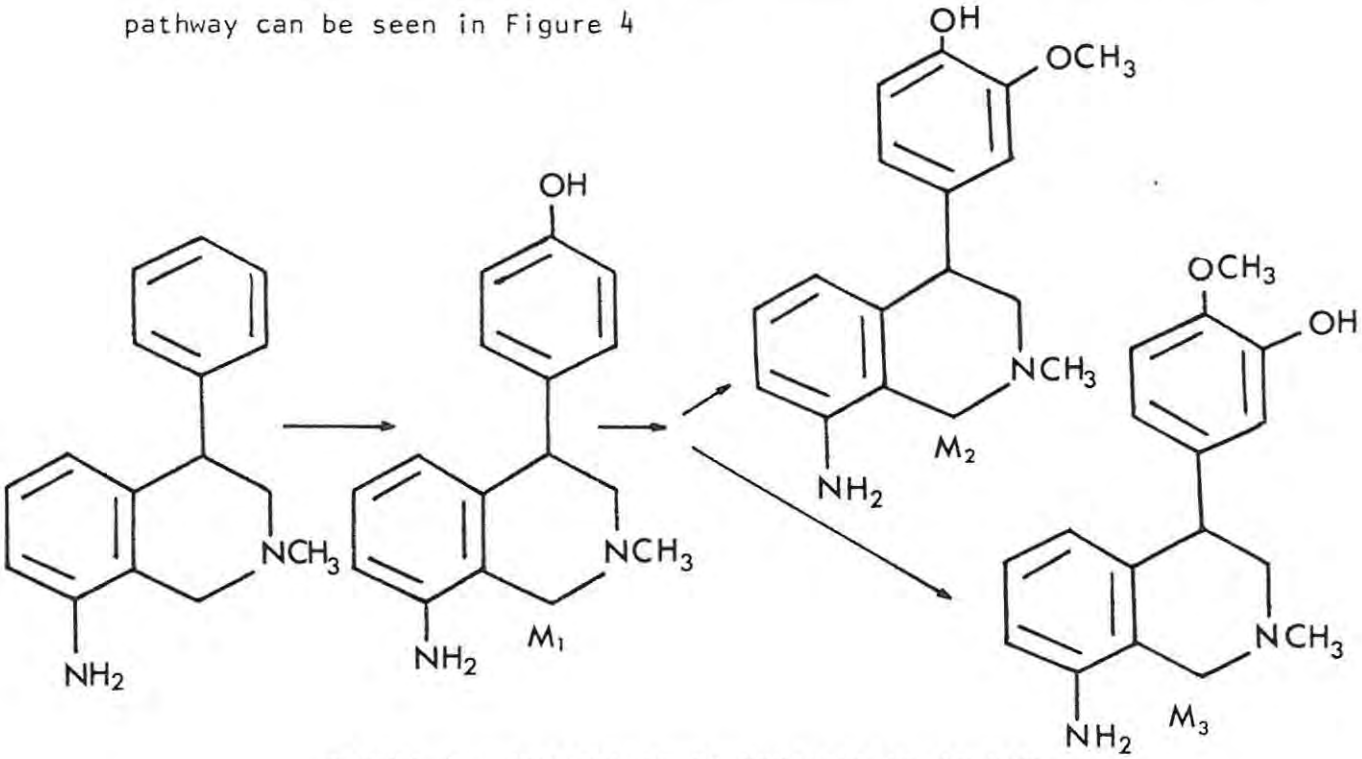


FIGURE 4 : METABOLISM OF NOMIFENSINE IN MAN

M_1 = 4 Hydroxyphenyl Nomifensine

M_2 = 3 Methoxy-4-hydroxyphenyl Nomifensine

M_3 = 3 Hydroxy-4-methoxyphenyl Nomifensine

SECTION 4

Drug - Receptor Interactions. Interaction of One or More Drugs with One Receptor System

4.1. Dose Response Curves

For an analysis of the action of drugs and of the relationship between structure and action, dose response curves are indispensable[15].

Theory

When the effect of a drug is studied in a simple isolated organ suspended in a bath fluid such as Ringer or Tyrode solution, the influence of drug transference, transport, chemical transformation, excretion, etc., is reduced to a minimum. The concentration of the drug in the bio-phase in the isolated tissue preparation, may be supposed to be equal or proportional to that in the bath fluid, which may be supposed to be equal to the concentration or dose added.

Receptors may be molecules, parts of molecules or molecule complexes. A single receptor can be occupied by only one molecule of the drug at a time.

The formation of a drug-receptor complex may imply a very temporary interaction between drug molecule and receptor; it may be just a slight contact between the drug molecule and receptor. On the other hand, there may be a definite, possibly a prolonged, chemical binding between them. The binding can be reversible or irreversible. Only reversible reactions will be discussed.

The relation between a drug, A, and the receptor, R, can be represented by:

$$[R] + [A] \underset{K_2}{\overset{K_1}{\rightleftharpoons}} [RA] \qquad (1)$$

Where [R] is the concentration of free receptors, [A] the drug concentration in the bio-phase, and $[R_A]$ the concentration of the drug receptor complex, that is, the quantity of drug bound to the specific receptors. The total concentration of receptors, [r] is $[R] + [R_A]$. An increase of the concentration of the drug will result in an increase of the quantity of the drug-receptor complexes. Since the number of specific receptors in the biological object is limited, the maximal amount of drug-receptor complex has a limit too.

As long as a gradual increase of the dose of the drug results in a gradual saturation of the receptor system, the relation between the fraction of receptors occupied by the drug and the concentration of the drug in the bio-phase is assumed to obey the Mass Action Law, or the adsorption isotherm of Langmuir.

In the case where the concentration of the drug is high compared to the number of specific receptors, it can be assumed to be constant. Then the fraction of the total number of receptors occupied by the drug A is represented by Equation 2.

E
|
R
↕
A

$$\frac{[R_A]}{[r]} = \frac{1}{1+(K_A/[A])} \qquad (2)$$

Where $K_A = K_2/K_1$. $[R_A]/[r]$ increases with the concentration of the drug, [A], and decreases with the dissociation constant K_A of the drug receptor complex R_A. Thus the 'affinity' of the drug for the receptor is proportional to the reciprocal of K_A. E represents the effect.

In order to induce an effect, a drug must interact with the receptor, that is, it must have an 'affinity' for the receptor. But this is not sufficient. It must interact with the receptors

in an 'effective' way; the drug must also have what is called an intrinsic activity.

In the simplest although not the most probable case, the effect will be proportional to the quantity of drug-receptor complexes formed. The intrinsic activity of the drug is a measure of its ability to contribute to the stimulus and in this simple case, to the effect. The stimulus is linearly proportional to the quantity of drug-receptor complex formed, but dependent on the intrinsic activity of the drug.

A certain value of the stimulus always results in the same effect. A certain quantity of drug-receptor complex, however, does not necessarily result in a constant stimulus. Here the intrinsic activity of the drug is determinant. The stimulus, S_A, expressed as a fraction of S_m, the maximal stimulus obtainable becomes

$$\frac{S_A}{S_m} = \frac{\alpha}{1+(K_A/[A])} \qquad (3)$$

If a linear proportionality between stimulus and effect is assumed, then the effect, E_A, induced in the effector, E, by a certain concentration of the drug, [A], as a fraction of the maximal effect, E_m, obtainable with a drug of this type, is represented by equation 4.

$$\frac{E_A}{E_m} = \frac{[R_A]\alpha}{[r]} = \frac{\alpha}{1+(K_A/[A])} \qquad (4)$$

The effect obtained with a certain dose of A, that is the activity of A, increases with the intrinsic activity, α, and with the affinity $1/K_A$. With high doses of A, the maximal effect for A, E_{Am} is reached and $E_{Am}/E_m = \alpha . K_A = [A]$ if $E_A = \frac{1}{2} E_{Am}$.

This gives us a mathematical equation for determining the values of the affinity and the intrinsic activity of a drug. The affinity is proportional to the reciprocal of that log concentration of the drug,

that gives a response equal to half the maximal response obtainable with that drug. The intrinsic activity of the drug that gives the highest response is taken as unity, then, relative values for the intrinsic activity of other drugs can be given.

The dose response curves as calculated from equation 4 are hyperbolic. On a log dose scale they take the character of a sigmoid curve. Figure 5 represents theoretical dose-response curves of various drugs calculated from equation 4. The drugs used have varied affinity as well as intrinsic activity.

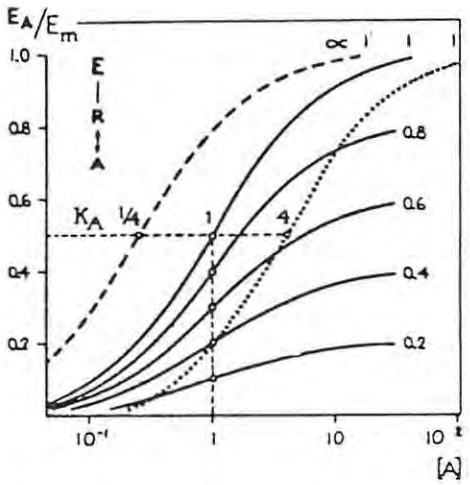


Figure 5. Theoretical log concentration response curves for compounds with varying values for the afinity ($1/K_A$) and the intrinsic activity (α). Note the parallel shift in the curves with a variation in K_A, and the decreases in the maximal height and in the shape, with a decrease in α.

4.2. Competitive Interaction

On the basis of the Mass Action Law, the effect, E_{AB}, of the combination of the drugs A and B competing for the same receptor system, R, may be expressed as a fraction of the maximal effect, E_m, as represented in equation 5.

$$\frac{E_{AB}}{E_m} = \frac{[R_A]\alpha}{[r]} + \frac{[R_B]\beta}{[r]} \qquad (5)$$

$$= \frac{\alpha}{1+(1+[B]/K_B)K_A/[A]} + \frac{\beta}{1+(1+[A]/K_A)K_B/[B]}$$

The contribution of A and B to their common effect $[A_B]$ is determined by their concentrations [A] and [B], their affinity to the receptors or the reciprocals of the dissociation constants K_A and K_B of the drug-receptor complexes R_A and R_B, respectively, and by their intrinsic activities α and β.

4.2.1. Competitive Antagonism

Theory

Suppose one of the drugs, B, has an intrinsic activity, $\beta = 0$ then the right term of equation 5 = 0.

Since competitive interaction exists at least two compounds must be involved. The easiest way to study the interactions of these compounds with respect to their biological effects is to make dose-response curves of one of the compounds in the presence of constant concentration of the other. This means that two sets of curves are possible.

1. A set of dose response curves for compound A in the presence of compound B in a concentration which is constant for each curve but varies for the various curves.
2. A set of dose response curves for compound B in the presence of compound A in a concentration which is constant for each curve but varies for the various curves.

The activity of an antagonist against an agonist can be tested in two ways.

1. The preventive one. In this case the antagonist is added first and prevents the effect of the agonist.
2. The curative one. In this case the antagonist is added while the agonist e.g. a spasmogen, is in action. The contraction induced then is reduced by the antagonist.

Most frequently one set of curves will be sufficient. Sometimes it will be useful to determine both as they may reveal different characteristics of the mode of action of the compounds.

Figure 6 represents theoretical dose response curves calculated from equation 5. Here dose response curves for A, a compound with an intrinsic activity, $\alpha = 1$, are calculated in the presence of constant concentration of B, a compound with an intrinsic activity, $\beta = 0$, B is a competitive antagonist of A. The presence of drug B results in a parallel shift of the log dose response curve for compound A to higher concentrations. The influence of B can be overcome by an increase in the dose of A. An increase in the dose of A always results finally in occupation of all receptors by this compound. The more of B that is present, the higher must be the dose of A in order to obtain an effect equal to E_{Am}.

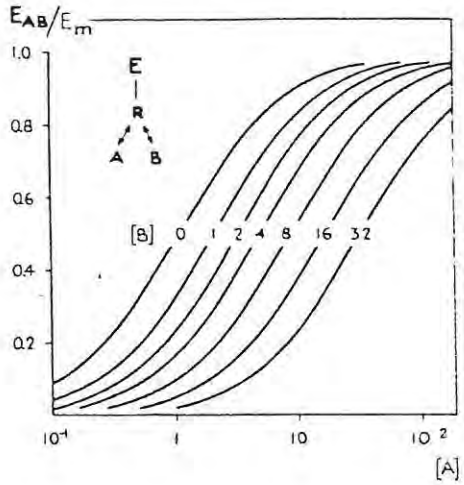


Figure 6. The theoretical concentration response curve for an agonistic compound A combined with various concentrations of a competitive antagonist B. (Equation 5. $K_A = K_B = 1$, $\alpha = 1, \beta = 0$). Note the parallel shift in the curves for A. Concentration in M^{-1}.

4.2.2. Evaluation of Agonists and Competitive Antagonists

The dose of an agonist, A, which produces an effect, E_A, that is 50% of the maximal effect, E_{Am}, that can be produced by the agonist, is called the ED_{50}. According to the theory:-

$$K_A = [A] \text{ if } \frac{E_{Am}}{E_A} = 2$$

Miller introduced the so-called pD values, the negative logarithms of the concentrations concerned in moles/litres. Ariëns used the nomenclature pDx, where x is equal to E_{Am}/E_A. The ED_{50} gives the pD_2 as an experimental value, which correlates with $-\log K_A$ from the theory, and therefore with the affinity of A for

the receptors.

Since K_A = [A] it can be said that these values equal dissociation constants. Dissociation constants are expressed as a positive value, i.e.

$$pD_2 - \log \text{conc}^n = \log \frac{1}{K_A}$$

The higher the pD_2 value, the greater is the affinity of the drug.

Competitive antagonists are better approached on the basis of the pAx values. In the case of competitive antagonism, the dose response curves for the agonist are shifted in a parallel fashion by the antagonist.

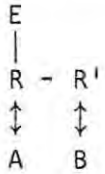
The negative logarithm of that dose of the antagonist that requires a doubling of the dose of the agonist to compensate for the action of the antagonist, is called the pA_2 value. If instead of a double dose, a tenfold dose of the agonist is needed, it is called the pA_{10} value etc. It follows from equation 5 that the pA_2 value is proportional to the negative logarithm of K_B.

Since K_B = [B]

$pA_2 = -\log$ [B]

pA_2 is therefore determined when [B] is such that a doubling of agonist is required to compensate for the action of antagonist B.

4.3. Non competitive Interaction



B interacts with receptors R', which are different from those for A, then the relation between A and B is a non-competitive one.

The contribution of drug A to the effect is changed by B. The occupation of the receptors R' by B as such, does not result in any effect. Only if A is present, can B change the response obtained with A. The degree to which this happens depends on the degree to which the receptors R' are occupied by B. This relationship is expressed in equation 6.

$$\frac{E_{AB}}{E_m} = \frac{E_A}{E_m} \left(1 + \frac{\beta'}{(K'_B[B]+1)}\right) \qquad (6)$$

B' is the intrinsic activity of B with respect to the change induced in the effect of A. The occupation of R' by B results in a virtual change in the intrinsic activity of A. K'_B is the dissociation constant of the drug-receptor complex R'B. The interference of B can result in an increase or a decrease of the response of the effector system. Then B' has to be supplied with a positive or negative sign respectively.

Theory

Depending on the value of the intrinsic activity β', the antagonism may be complete or partial. If -1 is substituted for β' for high doses of B, E_{AB}/E_m becomes zero. Hence there is complete antagonism. Figure 7 represents the theoretical dose-response curves calculated from equation 6, by substituting -1 for β'. In the case of competitive antagonism the effect is determined by the relation between the concentration of agonist and antagonist; the antagonism is surmountable.

In the case of a non-competitive antagonism the effect is determined by the concentration of the antagonist only. There is no shift in the curves but a gradual decline and disappearance of the effect at high concentrations of B. The non-competitive antagonistic

action is surmountable; this is true if high doses of the agonist or high doses of the antagonist are used. If lower doses of both the agonist and antagonist are involved, the decrease in the effect caused by the antagonist may be abolished to a certain degree by an increase of the dose of the agonist. In that case, the non-competitive antagonist is surmountable to a certain degree.

If a value between 0-1, say 0,5, is substituted for intrinsic activity β', with high doses of B the second term of equation 6 becomes 0,5 and the effect of A is reduced by 50% of its original value. Then there is a partial non -competitive antagonism.

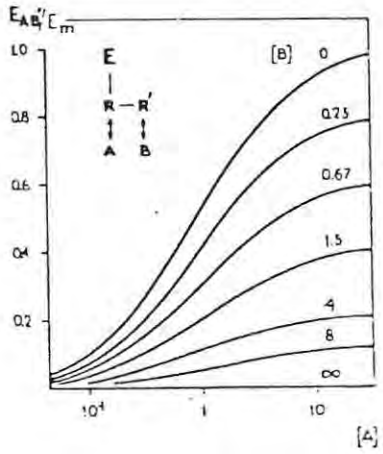


Figure 7. Theoretical log concentration-response curves for the agonist A combined with various concentrations of the non-competitive antagonist B. (Equation 6 $K_A = K_B' = 1, \alpha = 1$. $\beta' = -1$) Concentration in M^{-1}

4.3.1. Evaluation of Non-competitive antagonists

For non-competitive antagonists a procedure analogous to that used for an agonist may be followed. In the case of the agonist, pD_x values are used. The pD_2 value corresponds to the negative

logarithm of that dose of the drug for which E_{Am}/E_A reaches a value of 2. From equation 6 it follows that if the effect E_A of an agonist A is reduced by a non-competitive antagonist B, of which the intrinsic activity β' is = -1, to 50% of its original value, and if $E_A/E_{AB'} = x = 2$, [B] is equal to K_B. This implies that the pD'_2 value which is - log [B] for x = 2, becomes equal then to - log K'_B. A criterion for the non-competitive inhibition, is that the pD'_x value of antagonist is independent of the original effect E_A.

Theories for the possible mechanism by which tricyclic antidepressives act as antagonists have now been discussed above.

For further theories on other competitive antagonisms, the reader is referred to Ariëns[15].

SECTION 5

THE EFFECTS OF DRUGS AND STEROID HORMONES ON THE ENZYMES OF THE ENDOPLASMIC RETICULUM

5.1 Drug Metabolism Concepts

The hepatic drug metabolizing enzymes are membrane bound and are located in the microsomal membranes of mammalian liver cells. These membranes are derived from the rough and smooth endoplasmic reticulum. These enzymes are involved in the oxidative metabolism of drugs, steroids and other hormones with biosynthesis of cholesterol[43,44], and its catabolism to bile acids[45,46], the oxidation of fatty acids[47,48] and the oxidation of prostaglandins[49].

The enzymes of the hepatic endoplasmic reticulum, which metabolize drugs and steroids are often referred to as the microsomal drug metabolizing enzymes, and are responsible for the oxidation, reduction and conjugation of drugs, other foreign compounds (xenobiotics) such as pesticides, food additives and industrial chemicals.

The hepatic drug metabolizing enzymes function as a multicomponent electron transport system[50,51]. The microsomal electron transfer reaction (Figure 8) reveals the function of at least three flavoproteins, two hemoproteins and iron containing protein.

The flavoproteins function as reduced pyridine nucleotide dehydrogenases and they participate in the transfer of reducing equivalent to cytochrome b_5 from NADH, via the flavoprotein (fp_1), NADH-cytochrome b_5 reductase, or from NADPH by flavoprotein (fp_2), NADPH cytochrome P-450 reductase[52]. The reduced flavoprotein, fp_2 can also undergo oxidation by ferric cytochrome P-450[53], to react with molecular oxygen giving rise to a superoxide anion for the initiation

of lipid peroxidation or heme degradation. A third flavoprotein (fp_3), participates in the oxidation of tertiary amines giving rise to N-oxides[54]. This fp_3 may also function in an oxygen dependent oxidation of sulfhydryl groups for the formation of disulfide bonds.

5.1.1 Proposed Cyclic Function of Cytochrome P-450

One postulated scheme illustrating the cyclic pattern of reduction and oxygenation of cytochrome P-450, as it interacts with substrate molecule, electron donors and oxygen is shown in Fig. 9.

Briefly, the reaction may be summarized as follows:

(a) The ferric hemoprotein (Fe^{+3}) can interact with a molecule of substrate (R) resulting in a complex ($Fe^{+3}R$). The orientation of the substrate molecule as it sits in proximity of the heme iron, the presumed binding site for oxygen, remains a conjecture although the role of steering groups on the substrate molecule or the influence of hydrophobic environment of the heme may be considered as possible directing forces.

(b) The substrate complex ferric-cytochrome P-450 ($Fe^{+3}R$) undergoes reduction to a ferrous cytochrome P-450 substrate complex ($Fe^{+2}R$) by electrons originating from NADPH and transferred by the flavoprotein (fp_2) NADPH-cytochrome P-450 reductase.

(c) Reduced cytochrome P-450 ($Fe^{+2}R$) can react with carbon monoxide to form a derivative which is readily identifiable spectrophotometrically by an absorbance band maximum at about 450 nm. Alternatively, reduced cytochrome P-450 can react with oxygen to form a complex termed oxycytochrome P-450 ($Fe^{+2}O_2$-R).

(d) Oxycytochrome P-450 ($Fe^{+2}O_2$-R) can presumably dissociate to give a superoxide anion (O_2) concomitant with the regeneration of the ferric hemoprotein. Alternatively, the complex of oxycytochrome P-450 may

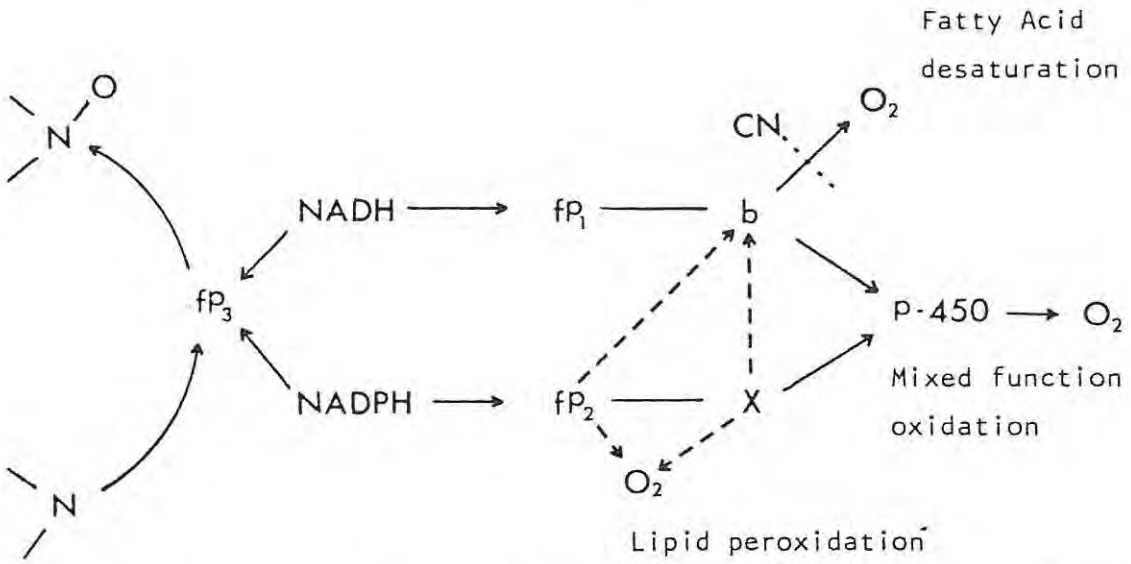


Figure 8. Schematic Diagram of Microsomal Electron Transport Reactions

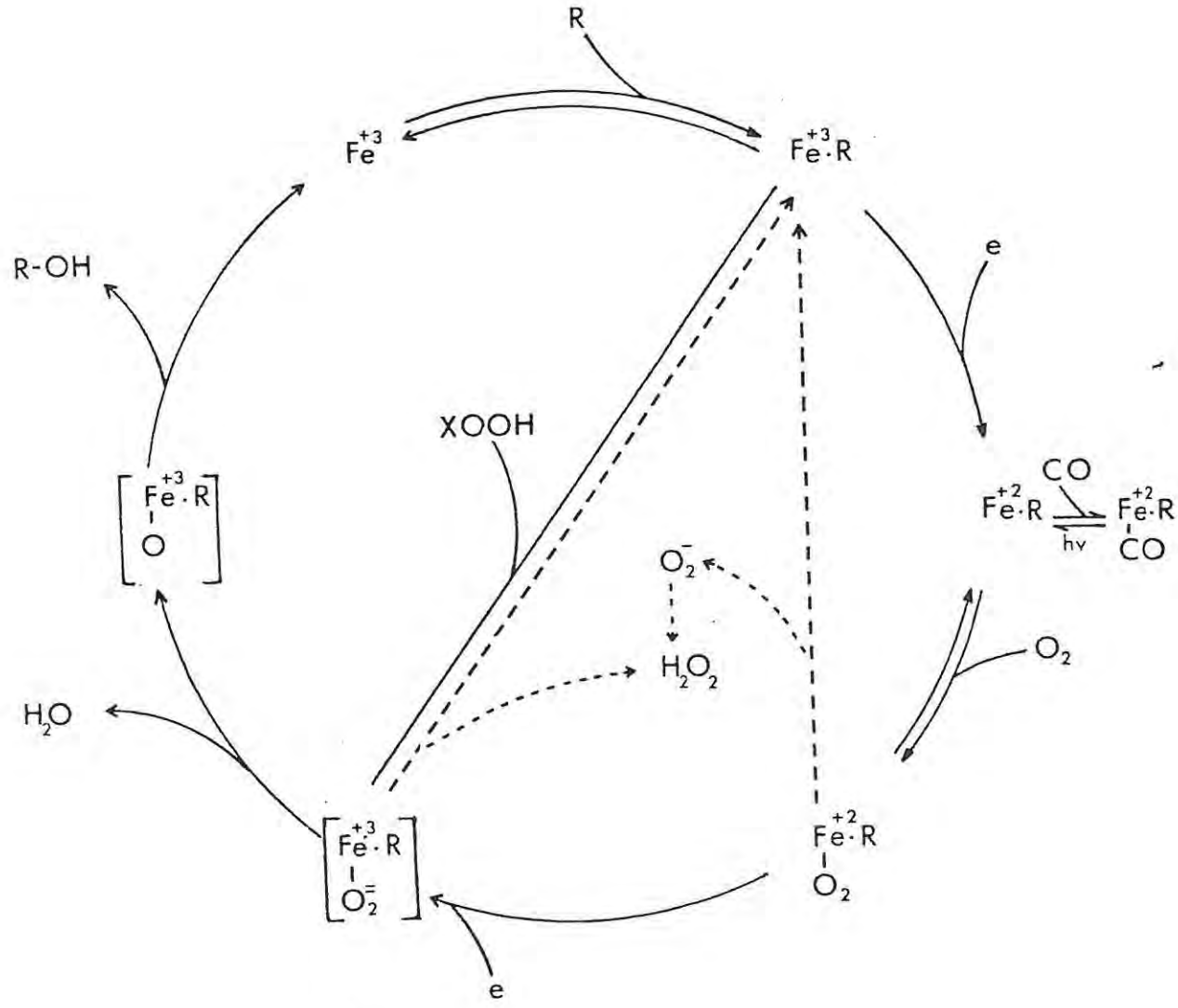


Figure 9. Schematic Diagram of the proposed Cyclic Function of Cytochrome P-450. The Substrate Molecule is Designated R. The Valence State of the Heme Iron of Cytochrome P-450 is indicated.

undergo further reduction to form the equivalent peroxide anion derivative of the substrate bound hemoprotein. Studies with the membrane bound cytochrome P-450 of liver microsomes suggest that a donation of a proposed second electron occurs via cytochrome b_5. This conclusion is the basis for explaining the synergistic effect observed during the concomitant oxidation of NADPH and NADH by this system.

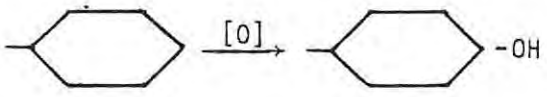
(e) The proposed peroxide anion complex of cytochrome P-450 may undergo protonation and dissociate as hydrogen peroxide or it may rearrange to form an oxime derivative concomitant with the release of water.

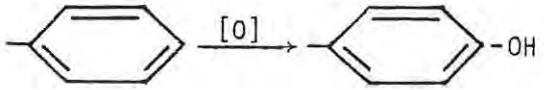
(f) Least understood is the mechanism of dissociation of the hydroxylated product and the restoration of the low spin form of ferric cytochrome P-450.

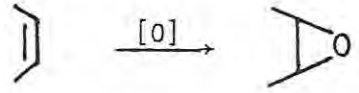
5.1.2 Drug Hydroxylations

It has been suggested that one single enzyme system is responsible for all the differing types of hepatic microsomal hydroxylations which may include the following[51].

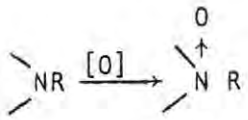
Aliphatic oxygenation $-RCH_3 \xrightarrow{[O]} RCH_2OH$

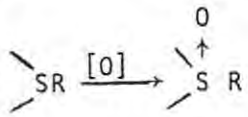
Alicyclic oxygenation 

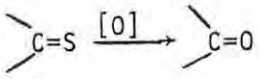
Aromatic oxygenation 

Epoxidation 

O and N-dealkylation $\rangle NCH_3 \xrightarrow{[O]} \rangle NCH_2OH \xrightarrow{[O]} \rangle NH + HCHO$

N-oxidation 

Sulphoxidation 

Desulphuration 

Furthermore, it is considered that this one enzyme system is responsible for the oxygenation of all the different substrates, including drugs and other foreign chemicals, steroids and fatty acids.

5.1.3 Cytochrome P-450 and Spectral Changes

To obtain an indication of the type of bonding between xenobiotic and cytochrome P-450 during drug metabolism by the mixed function oxidase system, spectroscopic studies can be applied.

5.1.3.1 Carbon monoxide Difference Spectrum

A difference spectrum is generated when a microsomal fraction is divided into two identical cuvettes, then placed in a dual beam spectrophotometer and a baseline run between 500 nm and 360 nm. A compound (in this case carbon monoxide) is added to the sample cuvette and the resulting spectrum measures the difference between the reference and sample cuvettes (see Figure 10).

The absorption spectrum of the reduced (Fe^{2+}) form when bound to carbon monoxide gives a maximum at a wavelength of 450 nm[52,53].

A smaller peak at 420 nm also exists because some P-450 is converted to a new complex with a maximum absorption at 420 nm[54].

Carbon monoxide competitively inhibits the enzyme since it competes with oxygen for the binding sites on reduced (Fe^{2+})

cytochrome P-450. Moreover, this inhibition is reversible with irradation with light at wavelength of 450 nm[55].

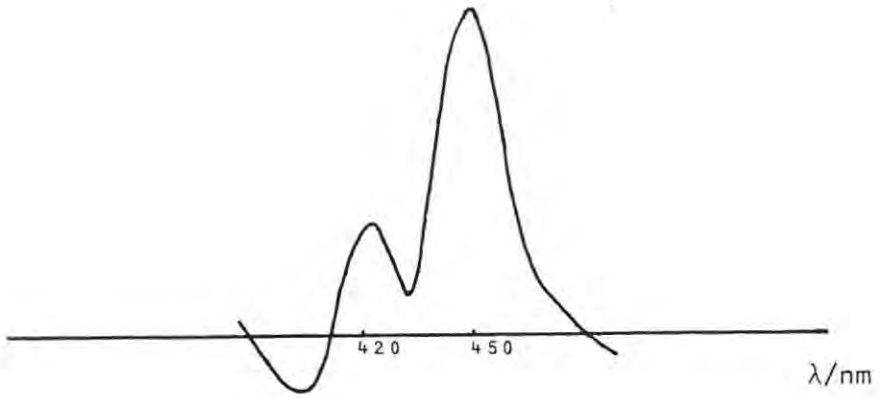


FIGURE 10. CARBON MONOXIDE DIFFERENCE SPECTRUM

5.1.3.2 Spectral Changes and Binding of Cytochrome P-450 to Drugs

The binding of many compounds to the microsomal mixed function oxidase system produces two distinct type of difference spectra. These spectra are termed type I and type II spectra. Compounds giving rise to type I and type II difference spectra with hepatic microsomes have come to be known as type I and type II compounds. A third, less common spectrum, a reverse type I, is also possible. The various types of spectra are depicted in Figure 11.

The binding of substrate to microsomal cytochrome probably precedes enzymatic oxidation, and the manifestation of a spectral change is regarded as evidence that an enzyme compound complex is formed. The type I spectrum has a maximum at 390 nm and a minimum at 420 nm (Fig. 11a). Compounds which give rise to type I include:- imipramine, testosterone, barbiturates, aminopyrine. The type II spectrum is characterized by a peak at approximately 425-435 nm and a trough at 390 nm (Fig. 11b).

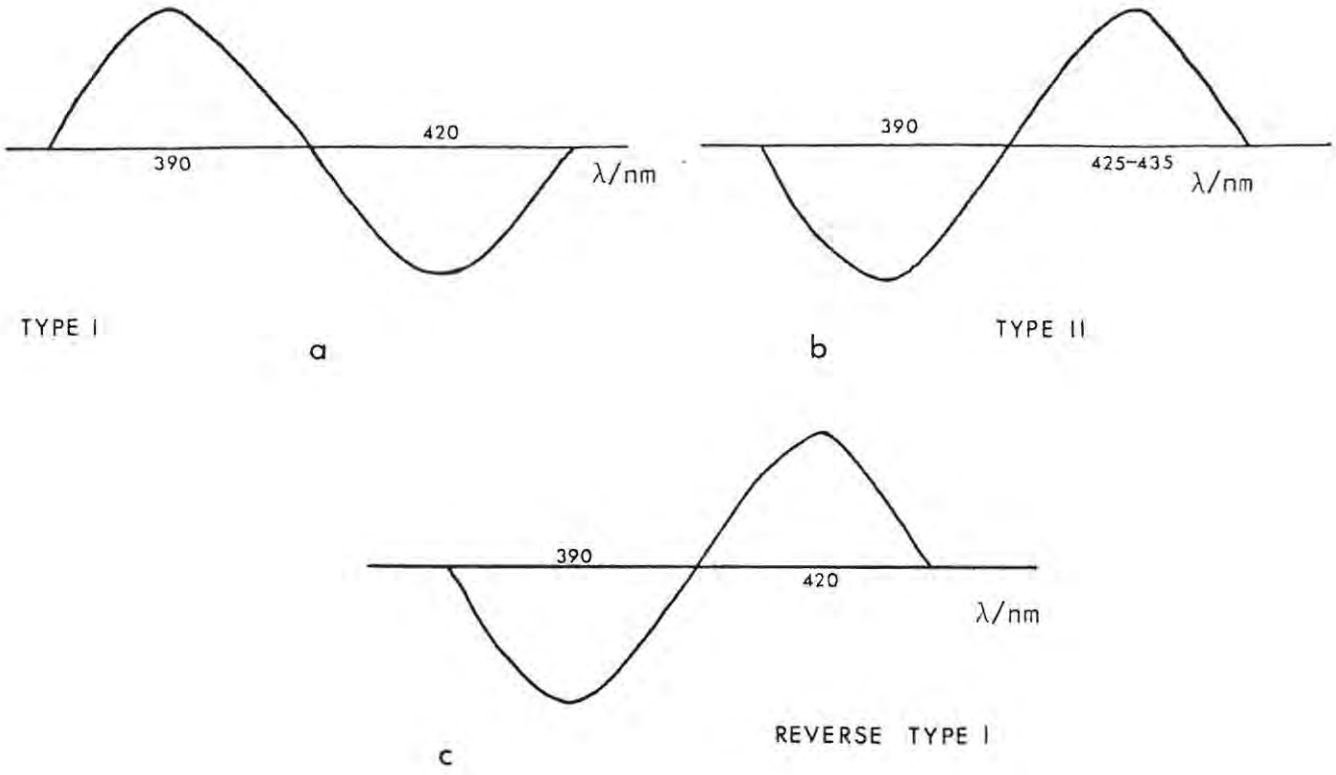


FIGURE 11. DIFFERENCE SPECTRA

Type II substrates normally contain a basic nitrogen atom and include compounds such as aniline, pyridine and 2,4-dichloro-6 phenylphenoxyethylamine hydrochloride. These compounds are generally not metabolized by cytochrome P-450. Evidence has shown that many compounds may combine in varying degrees with both binding sites and this may explain the metabolism observed with some type II compounds[56,57].

The reverse type I (RI) spectrum (also known as modified type II) has a peak at 420 nm and a trough at 392 nm (Fig. 11c). The cause of this spectrum has been explained and one explanation is that compounds (methanol, ethanol, 1-butanol, acetone and phenacetin) which elicit such a spectrum do so by displacing endogenous substrates from cytochrome P-450[58], i.e., the RI spectral change is caused by reversal of structural state of P-450 caused by prior binding of

endogenous substrate to the enzyme *in vivo*.

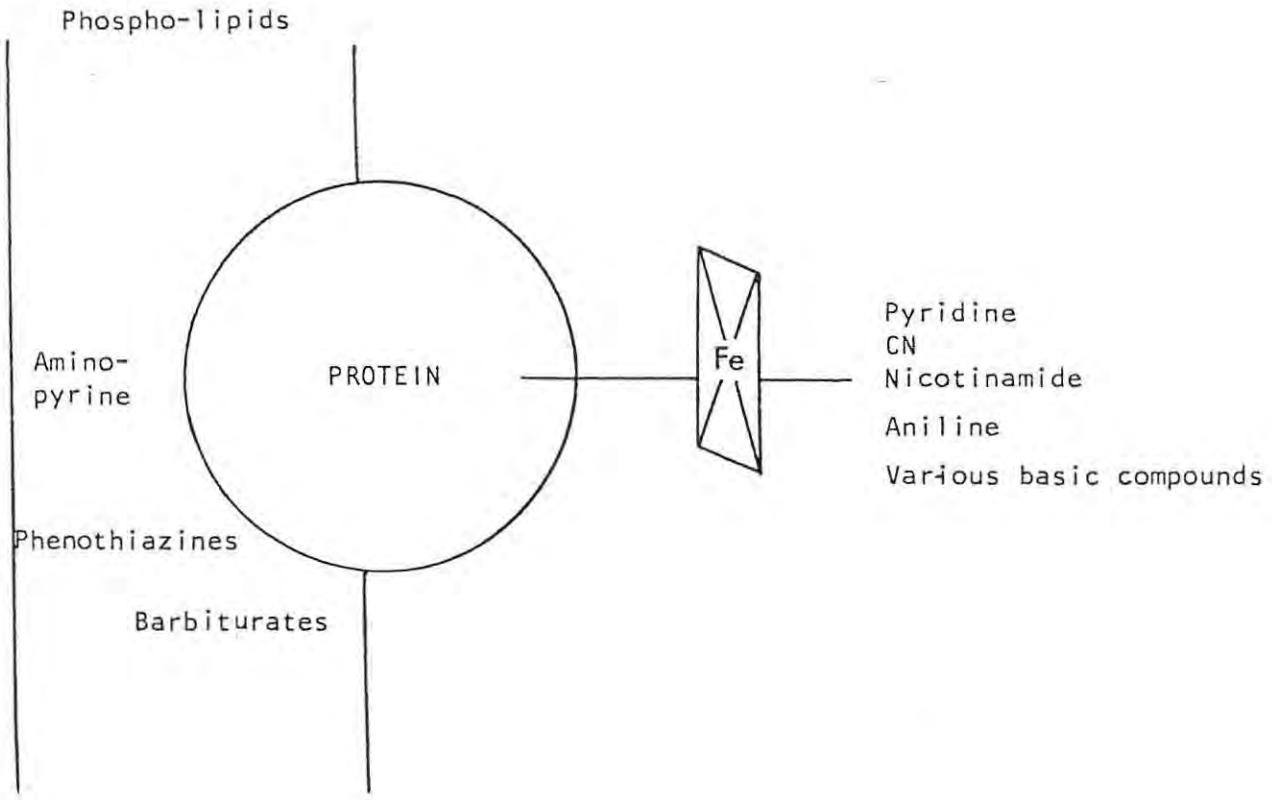
Some reverse type I substrates are metabolized. One proposed metabolism is that they are bound first to the type I site, then with increasing concentration to another site as well, giving rise to a new spectrum which obscures the type I component[57, 59].

Binding of a type I compound is thought to change the conformation of the protein, so that the Fe-binding shifts from the sulphur of one amino acid of protein to a nitrogen of a nearby amino acid. This shift from sulphur to nitrogen might cause the type I difference spectrum. Type II compounds are thought to bind as ligands to the 6 position, which is the site for oxygen i.e., ferric haemochrome formation.

Binding of type I and type II compounds may occur as depicted in Figure 12.

5.2 The Biological Function of the Hepatic Microsomal Drug Metabolising Enzymes

Drug metabolism occurs in two phases. Phase I is commonly an oxidation, a reduction or hydrolysis. Phase II is always a conjugation reaction. The purpose of drug metabolism is to convert the compound into a form which is more water soluble. The easiest way of achieving this is by conjugation with for example, glucuronic acid in a phase II reaction. But for conjugation to occur there must be a group in the molecule which can react with the conjugating agent. In general, this means that an ionic group is needed. Many drug molecules possess no such group and the purpose of a phase I reaction is to introduce such a group.

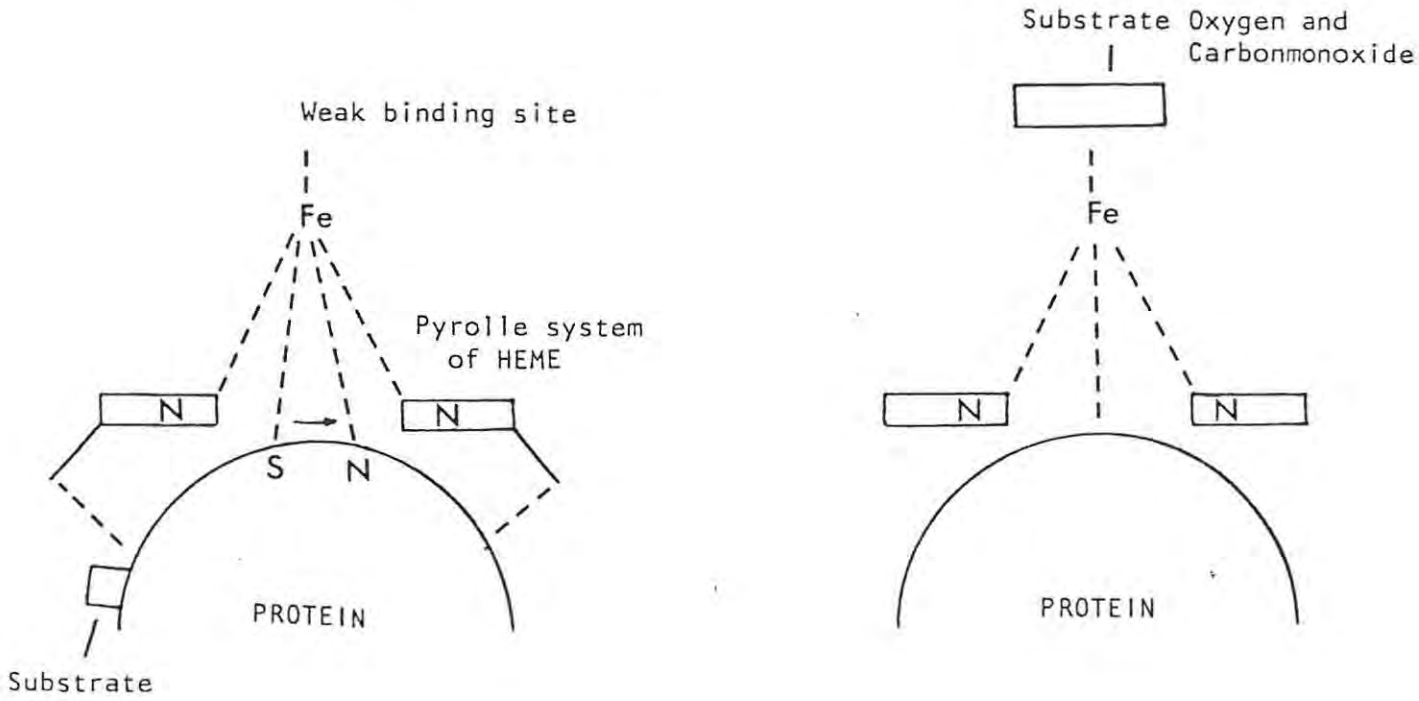


TYPE I

Binding to a Hydrophobic Region on Proteins

TYPE II

Binding to the 6th Ligand



TYPE I BINDING

TYPE II BINDING

FIGURE 12. PROPOSED BINDING SITES OF TYPE I AND TYPE II COMPOUNDS

The low Michaelis constant which characterizes the hydroxylation of testosterone, progesterone and oestradiol by hepatic microsomal enzymes supports the concept that the endogenous steroids are the normal physiological substrates and indeed preferential substrates of the oxidative drug metabolising enzymes[60].

5.3 The Effect of Drugs and Foreign Compounds on the Hepatic Drug-Metabolising Enzymes

The increased activities of the hepatic microsomal drug metabolising enzymes following the treatment of animals with a wide variety of drugs, pesticides, food additives, polycyclic hydrocarbons and other foreign compounds is now extremely documented. Stimulation of these enzymes occurs only when the inducing compounds are administered to the living animal or are perfused through isolated liver[61], and addition to preparation of the microsomal enzymes *in vitro* produces no stimulatory effect.

The drugs and foreign compounds which produce stimulation of these enzymes have widely differing pharmacological activities, and the only factors which they would all seem to have in common, are that they are lipid soluble and hence become localized in the endoplasmic reticulum of the liver, and are substrates of the microsomal mixed-function oxygenase.

5.3.1 Mechanisms of Induction of Hepatic Drug Metabolising Enzymes by Drugs and Polycyclic Hydrocarbons.

The first enzyme that increases during induction is δ-amino levulinic acid synthetase, which is involved in the synthesis of heme[51]. This is followed by cytochrome P-450 and the specific flavin enzyme for electron transport. All the other enzymes not

involved in drug hydroxylation increase later on to a much smaller extent. This is the case for cytochrome b5 and the relevent unspecific esterase[62].

If treatment with an inducing agent is not terminated the increase in cytochrome P-450 continues until a maximal amount is formed after three to five days, and a new steady state is achieved. So long as the inducing agent is present in the liver cells it enhances the rate of synthesis of the heme as well as of the protein moiety by causing an increase in messenger RNA formation in the nucleus[63].

Induction of induced enzymes is completely prevented if either actinomycin D, which acts at the level of transcription, or cycloheximide or puromycin, which act at the translation level, are concomitantly administered with inducing agent. Gerlehrter (1973)[64] mentions that stimulation of enzyme actively becomes insensitive to actinomycin D during treatment with inducer, and Nebert and Gilboin (1970)[65] found that the inducing system became insensitive to actinomycin D, but continued to be cycloheximide sensitive, i.e., protein synthesis is required continuously. These two processes for induction appear necessary:-

(a) Synthesis of induction specific RNA which is cycloheximide insensitive and thus translation independent.

(b) Translation related to the induction specific RNA which is insensitive to inhibition by actinomycin D and thus transcription independent.

The induction specific RNA may accumulate at the nuclear site bound to DNA. This accumulation of RNA causes the induction process to pass from the transcription-dependent to the transcription-

independent stage. The second, the translational step, involves the synthesis of either enzymes, protein, or a rapidly synthesized and degraded protein activator of the enzyme complex.

A significant enhancement of drug metabolizing enzymes is visible with an electron microscope. The smooth (but not the rough) membranes in the liver cells increase considerably. With the growth of the endoplasmic reticulum the liver becomes larger, predominantly by hypertrophy. This does not mean that any enlargement of the endoplasmic reticulum is necessarily associated with an increase in drug metabolizing enzymes. The magnitude of induction is also highly dependent on the age of the animals. One tenth of the phenobarbital dose in weaning rats produces nearly the same induction as the full dose in adult animals [66].

Observations during therapy and clinical studies show that an increase in drug metabolism can occur in man also [67]. However, the number of drugs known so far to act as inducers of the hydroxylating system during therapy is small; these include barbiturates, glutethimide, meprobamate, phenylbutazone, phenytoin and the antifungal agent griseofulvin. This list is far from complete. The inducing action of these drugs in man, however, is exceedingly inconsistent and many patients treated with the same compound at the same dose levels did not respond with increased drug metabolism [68].

5.3.2 Conditions Necessary for Induction

(1) The majority of lipid soluble compounds metabolized by the microsomal oxygenase have some inducing properties. This is true even for ethanol and for those drugs such as barbital which cannot be hydroxylated in measurable quantities during the period of testing. Alkaloids, however, seem to have no inducing capacity.

(2) The inducing action is dose dependent. However, many drugs are active only at nearly toxic doses; a few, however, such as long-acting barbiturates can induce the enzyme system when therapeutic doses are taken.

(3) Most important is the maintenance of a high concentration in liver cells for a certain time.

5.4 The Metabolism of Steroids by the Endoplasmic Reticulum

Many tissues other than the liver, for example the adrenal cortex, the testicular interstitial tissues, the corpus luteum, and the placenta, which are concerned with the biogenisis of the steroid hormones, have been shown to contain well developed endoplasmic reticula and microsomal enzymes, which are involved in metabolic pathways of cholesterol and steroid hormones.

The endoplasmic reticulum of the liver contains a number of enzymes which further metabolize and deactivate the steroid hormones, as these include the 2β, 6α, 6β, 7α, 16α hydroxylases and the 4,5αΔ^4 oxidoreductases [69,70]. These enzymes probably catalyse the first step of the conversion and have been shown to require $NADPH_2$ and oxygen and to be inhibited by carbon monoxide and an antibody to NADPH cytochrome-C-reductase [45,46].

5.4.1 Metabolism of Oestrogens

The metabolism of oestrogens is outlined in Figure13. Oestrone and oestradiol are interconvertible by a 17β-hydroxysteroid dehydrogenase. The conversion of oestradiol to oestrone appears to be more rapid than the reduction of oestrone to oestradiol and oestrone probably serves as the precursor of hydroxylated metabolites rather than oestradiol. One of the main metabolic pathways for oestrone appears to be hydroxylation to 16α-hydroxyoestrone followed by reduction to oestriol. Some of the

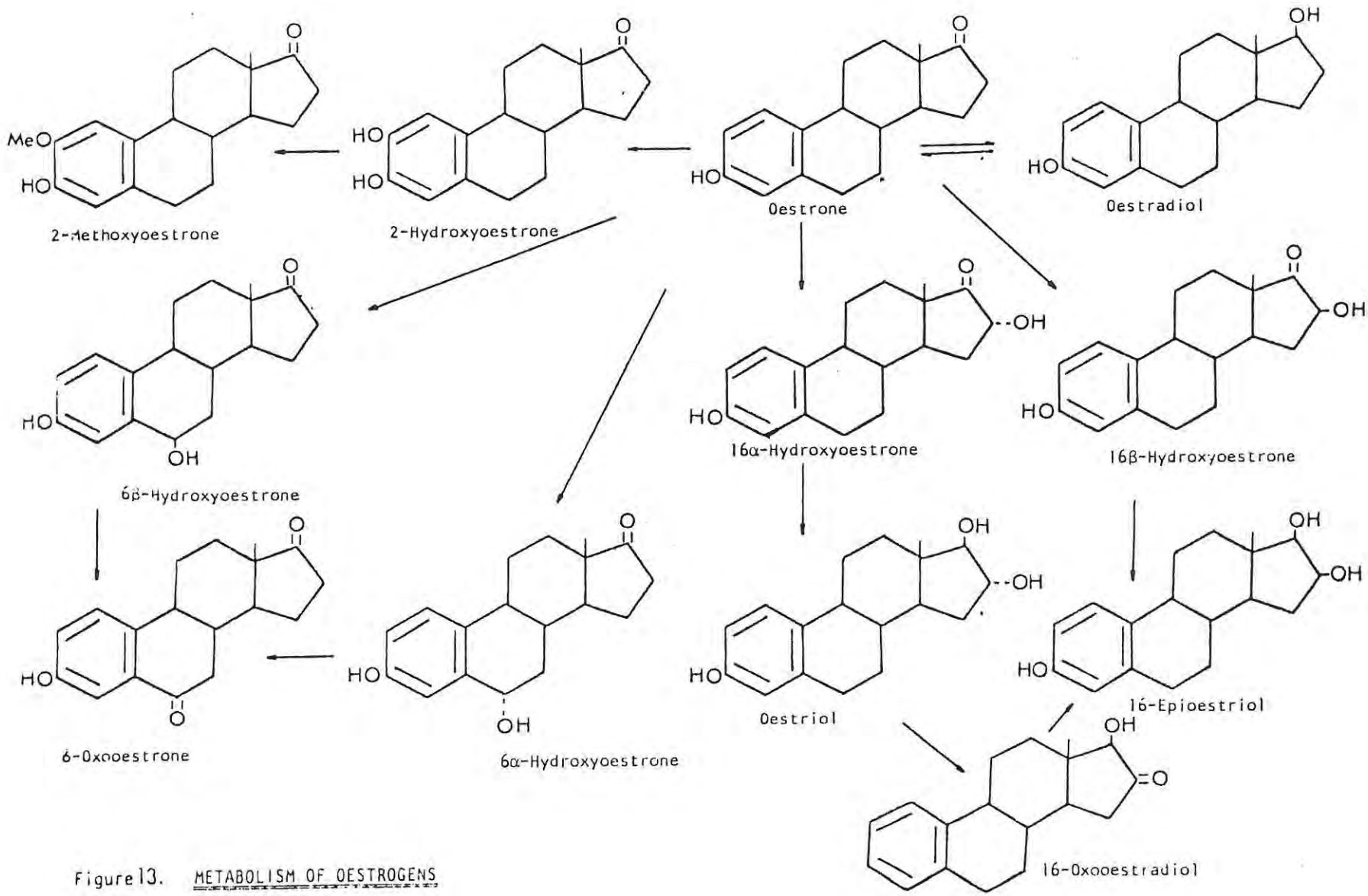


Figure 13. METABOLISM OF OESTROGENS

oestriol produced is oxidized to 16-oxooestradiol and this can undergo reduction to 16β-hydroxyoestradiol (16-epioestriol). Some 16α-hydroxyoestrone can be reduced by the enzyme 17α-hydroxy steroid dehydrogenase with the formation of 17-epioestriol but this does not appear to be quantitatively significant. The second important pathway appears to be by hydroxylation at position 2 with the formation of the catechol 2-hydroxyoestrone. The 2-hydroxy compounds are readily converted to 2-methoxy oestrogens by an O-methyl transferase. Demethylation of the 2-methoxyoestrogens occurs to only a slight extent. The 6α-and 6β-hydroxylases result in the formation of 6α- and 6β-hydroxyoestrone and 6α- and 6β-hydroxyoestradiol.

After administration of [^{14}C] oestrone or oestradiol, between 50% and 80% of the radioactivity is excreted in the urine within four to six days and up to 18% may be found in the faeces. Since the faecal excretion is much smaller, a large amount of the metabolites excreted in the bile, is reabsorbed and enterohepatic circulation of oestrogens occurs. The metabolites in the urine appear to be excreted mainly as glucuronides although sulphates exist [71]. Except in a few instances unconjugated metabolites occur in very small amounts. About 20% of the radioactivity in the extracted urine consists of oestrone, about 20% oestriol and a further 20% consists of 2-hydroxyoestrone. Smaller amounts (about 5%) of 2-methoxyoestrone are also present.

5.4.2 Metabolism of Progesterone

The major metobolic pathways undergone by progesterone are shown in Figure 14. The action of the 4-ene-reductase leads to the production of 5α-and 5β-pregnanediones which are then reduced by

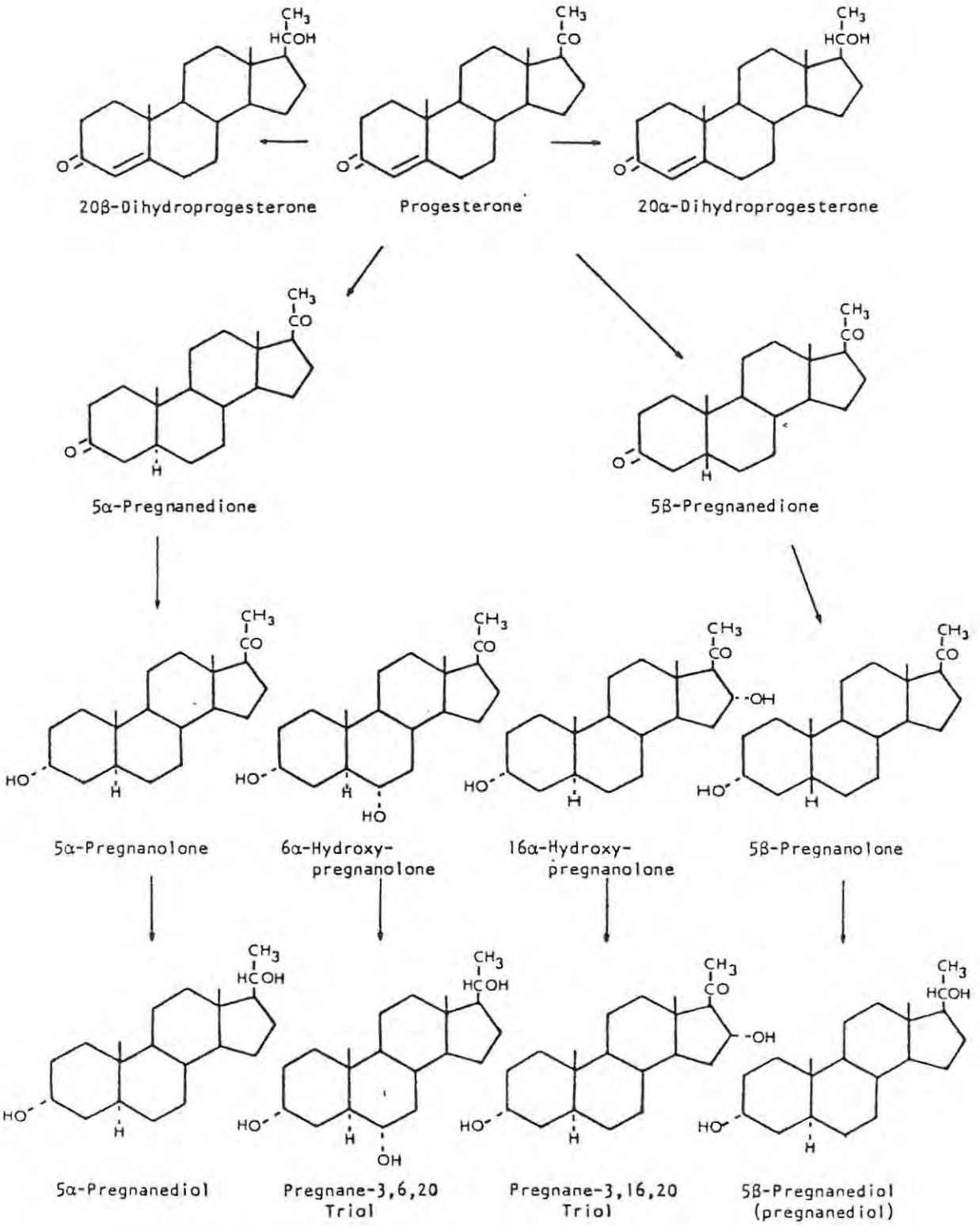


Figure 14. METABOLISM OF PROGESTERONE

the 3α-hydroxy and 3β-hydroxy steroid dehydrogenases. The former is much more active than the latter so that 3α-hydroxy steroids are formed predominantly. A number of tissues contain 20α-hydroxy and 20β-hydroxy steroid dehydrogenases of which the former is much more active in humans. As a result of these enzymes the C-20 oxo group is reduced to hydroxy; these enzymes appear to utilize either progesterone or the pregnanolone as substrates. By the action of the reductases and dehydrogenases eight possible isomers of pregnanediol can be produced but only three of these are of any importance. 5β-Pregnane-3α,-20α-diol is by far the most important single metabolite of progesterone. About 5% of administered progesterone is excreted in the form of pregnanolones and up to 2% as pregnanediones. Hydroxylation reactions are more important in the metabolism of progesterone than in the metabolism of androgens and up to 7% of the dose may be converted to metabolites containing a 6α-hydroxy group while possibly another 5% is converted to metabolites containing a 16α-hydroxy group [71]. Progesterone itself does not appear in urine except for very small amounts detected in late pregnancy. After the administration of [^{14}C] progesterone between 50% and 60% of the dose is excreted in urine; up to about 10% may be excreted in the faeces. Since about 30% is excreted via the bile it is evident that considerable reabsorption must occur.

5.4.3 Metabolism of Androgens

Since testosterone and androstenedione are interconvertible the metabolism of these two compounds is considered together. The main metabolic pathways are shown in Figure 15. The 17-oxo steroids and 17β-hydroxy steroids are interconvertible by the enzyme 17β-hydroxy steroid dehydrogenase; although the 17-hydroxy steroids have a greater

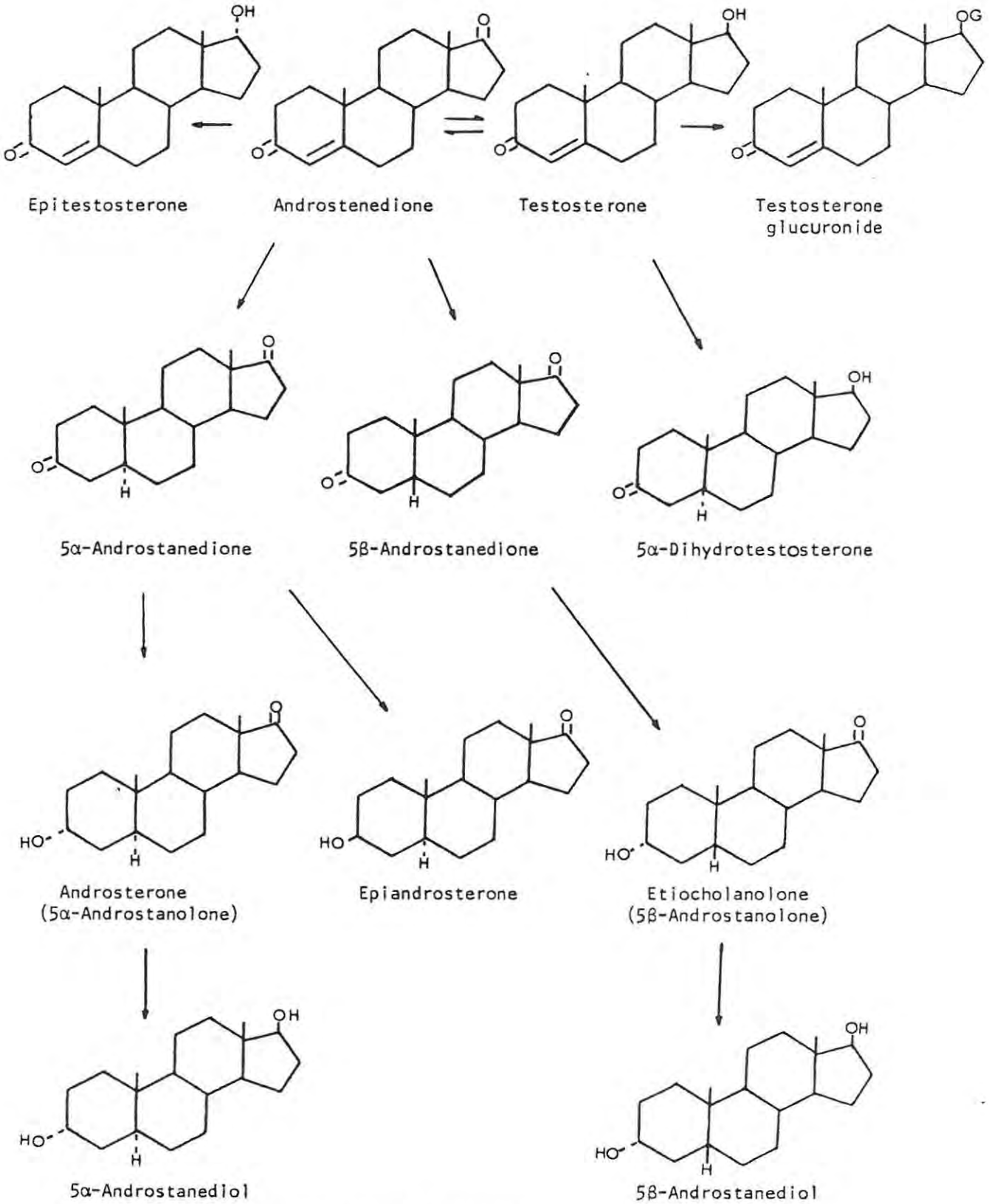


Figure 15 METABOLISM OF ANDROGENS

biological activity than the 17-oxo steroids, the 17-oxo steroids predominate in urine. The first stage in metabolism is reduction of the double bond in ring A carried out by the 4-ene-5α and 4-ene-5β reductases, which seem to have some substrate specificity. Reduction of this double bond with the production of 5α- and 5β-androstenedione leads to a marked reduction in the biological activity of the steroid. 17β-Hydroxy-5α-androstan-3-one (5α-dihydrotestosterone) is important since this compound has considerable biological activity and seems to be produced from testosterone in target organs from the male sex hormones [71]. A number of tissues contain both 3α-hydroxy and 3β-hydroxy steroid dehydrogenases; the activity of the former seems to predominate and more 3α- than 3β-hydroxy steroids are formed. After administration of labelled testosterone or androstenedione the greater part of the urinary radioactivity was associated with 5α-androstanolone (androsterone) and 5β-androstanolone (etiocholanolone). The percentage conversion of androstenedione or testosterone to urinary metabolites was approximately as follows: androsterone, 20%; etiocholanolone, 26%; 3β-hydroxy-5α-androstan-17-one, 1%; 5α-androstanediol, 1% and 5β-androstanediol, 1,5%. 6-Hydroxy and 19-hydroxy derivatives of testosterone and androstenedione are known although under most circumstances pathways leading to these compounds are minor ones. Small amounts of 17α-hydroxy-androst-4-en-3-one (epitestosterone) are produced, presumably from androstenedione by the action of a 17α-hydroxy steroid dehydrogenase. In addition to glucuronide conjugates, sulphate conjugates are also known in the case of C-19 steroids and most of the 3β-hydroxy-5α- and 5β-steroids are predominantly conjugated with sulphuric acid. The 3α-hydroxy

steroids appear mainly as the glucuronides although some sulphate formation takes place.

After administration of [^{14}C] testosterone, about 90% of the dose is excreted in urine, and about 6% appears in faeces. Since about 14% of the dose is excreted in the bile some reabsorption of the steroids from the gastro-intestinal tract must take place.

5.4.4 Metabolism of Synthetic Oestrogens

Only ethinyl oestradiol will be discussed here, the reader is referred to reference [71] for further information on mestranol, quinestral, and 17α-methyloestradiol.

The in vivo metabolism of 17α-ethinyl oestradiol in man has been thoroughly investigated; it was found that the elimination rate and the type of conjugation was approximately the same as for natural oestrogens [72]. It was found that in man 17α-ethinyl oestradiol reacts to form numerous metabolic products, the most important of which are 2-methoxy-17α-ethinyl oestradiol, 2-hydroxy-17α-ethinyl oestradiol-3-methyl ether, and D homoestradiol-17β (Fig.16).

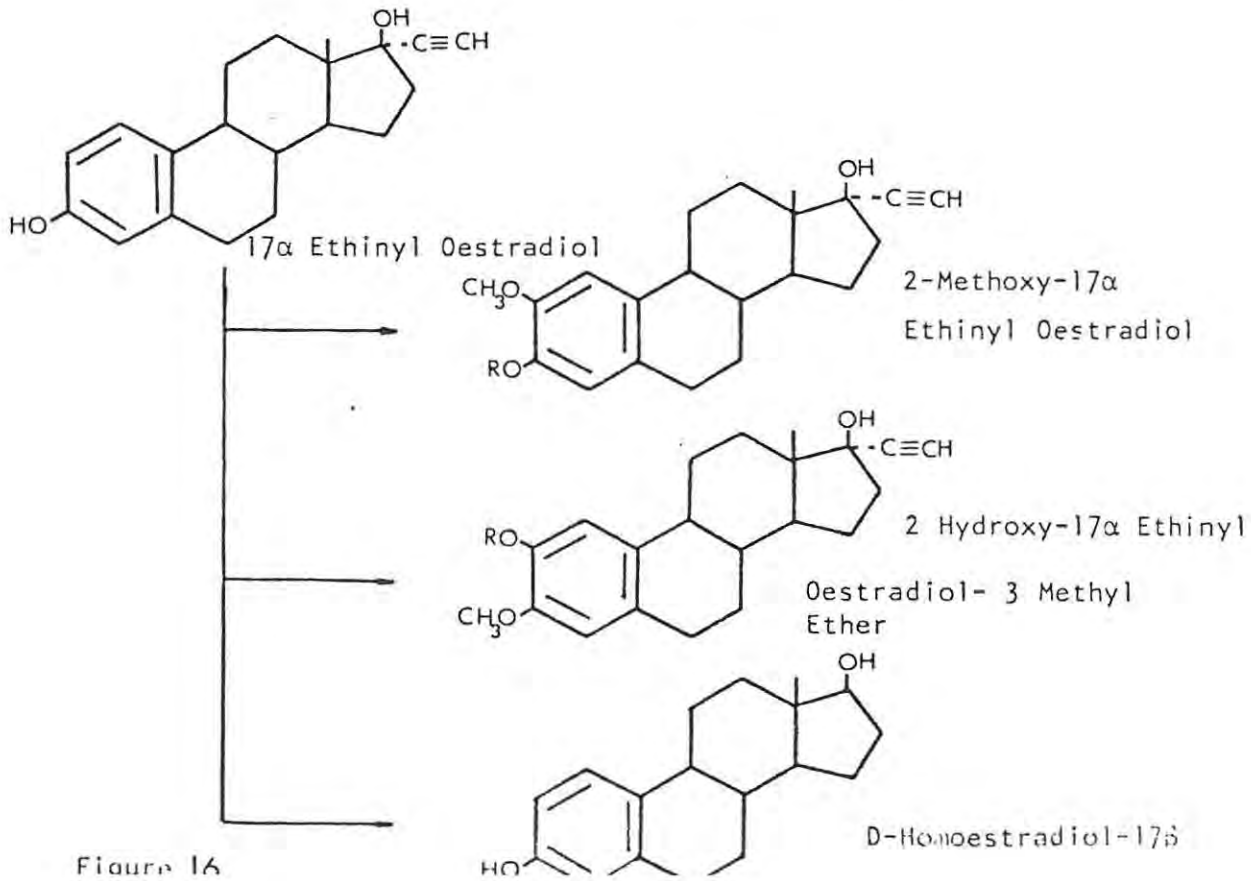


Figure 16

5.5 The Effects of Steroids on Hepatic Drug Metabolism

Steroids of all kinds, the endogenous corticosteroids, androgens, oestrogens and progestogens and various synthetic steroids have been shown to affect the metabolism of drugs by altering the activity of the drug metabolizing enzymes of the liver[67]. Table 5 shows the similarities between the hepatic microsomal enzymes which hydroxylate drugs and steroids[73].

TABLE 5 SIMILARITIES BETWEEN HEPATIC HYDROXYLASES THAT METABOLIZE STEROIDS AND DRUGS

(1)	Localized in liver microsomes; require NADPH and oxygen for activity.
(2)	Activity is higher in male than female rats, with little or no difference in mice.
(3)	Activity is higher in mammalian liver but lower in fish liver.
(4)	Activity is increased by pretreatment with drugs, pesticides and steroids.
(5)	Activity is inhibited by *in vitro* addition of SKF 525-A or Chlorthion
(6)	Drugs and steroids exhibit competitive inhibition with each other *in vitro*.
(7)	Activity shows a diurnal variation with minimal drug metabolizing activity coincident with maximum plasma levels of corticosteroid.

Since drugs and steroids would appear to be alternative substrates for the same hepatic microsomal mixed function oxygenase system, they might be expected to exhibit competitive inhibition of the enzyme system *in vitro*, and within a short period after

administration *in vivo*, though after a longer interval should give rise to an increase in the enzyme activity.

It has long been known in the rat that the duration and intensity of drug action is greater in the adult female than in the male. This difference of activity is due to an enhanced level of the hepatic microsomal drug metabolizing enzymes in the adult male rat and must be produced by the male sex-hormones for it only appears at puberty and may be abolished by castration[73]. This sex difference has not been observed to the same extent in other animals. Testosterone administered to female rats increases the rate of metabolism of many drugs and conversely the opposite effect is produced by the administration of oestrogens to the male animal[67]. Mice behave somewhat differently, a single dose of testosterone to female mice producing a biphasic effect, initially prolonging the action of hexobarbital and after a few days reducing this. Long term pretreatment of female mice with testosterone reduces the pharmacological activities of hexobarbital and chlorzoxazone by increasing their metabolism[74].

Cortisol, progesterone, testosterone, androsterone and oestradiol-17β all competitively inhibit the *in vitro* hydroxylation of hexobarbital and the N-demethylation of ethinyl morphine by rat liver microsomes, consistent with the view that steroids and drugs are alternative substrates for the hepatic microsomal mixed-function oxygenase system[75].

5.6 The Effect of Pregnancy on the Metabolism of Drugs

There has been a growing opinion among clinicians that pregnancy in humans imposes an inhibition on the ability to metabolise and deactivate drugs[76]. During pregnancy there is a considerable

increase in the formational circulation of the female sex steroid hormones, both progestogens and oestrogens, and since these steroid hormones may be metabolized by the same hepatic microsomal enzymes which metabolize drugs, it is possible that they may competitively inhibit the metabolism of drugs.

It has been shown that in pregnant rats and rabbits, and in animals pretreated with progesterone, the glucuronide conjugation of a number of substrates was markedly inhibited[77]. Sulphate conjugation is also inhibited during pregnancy and it has been suggested that this is due to the high levels of oestrogen[78].

5.7 The Effect of Oral Contraceptive Steroids

Since endogenous steroid hormones may act as competitively inhibiting substrates for the hepatic drug-metabolizing enzymes, and both natural oestrogens and progestogens of pregnancy have been shown to be associated with inhibition of these enzymes, it may follow that oral contraceptive steroids could also have an inhibitory effect which, in view of the extensive and prolonged usage of these compounds, might be highly undesirable. It has been shown that one hour after administration of norethynodrel to rats the *in vitro* metabolism of both hexobarbitone (a type I substrate) and of zoxazalamine (a type II substrate) was inhibited, whereas 24 hours later the metabolism of both compounds was increased[78]. The oestrogen mestranol administered to mice daily for 4 days inhibited the metabolism of hexobarbital and pentobarbital and increased the sleeping times produced by these barbiturates; on the other hand lynestrenol, a progestogen similarly administered, enhanced the metabolism of these barbiturates[79,80] and also that of phenytoin[80]. With acute treatment (2 hours after dosage) norethynodrel and ethinyl oestradiol inhibited the *in vivo*

metabolism of pentobarbital in rats. Whereas after chronic treatment (30 days) this *in vivo* metabolism was significantly increased[81].

In summary it would appear that many of these contraceptive steroids are acceptable substrates for the hepatic microsomal drug metabolizing enzymes, giving rise to modest inhibition of drug metabolism *in vitro*, and variable extents of induction of the various microsomal drug metabolizing enzyme activities *in vivo*, which on prolonged treatment finally return to approximately normal levels. The long term use of these oral contraceptive steroids thus appears unlikely to result in any major changes in the levels of the hepatic drug metabolizing enzymes, and hence is unlikely to result in any marked changes in the activity of drugs.

5.8 Sex Hormonal Alteration of Imipramine Response

5.8.1 Imipramine-Ethinyl Oestradiol in Women

Depression is more common in women than in men. It appears that men respond more promptly than women to imipramine. Some androgens could not be given to women because of masculinization, so as to enhance imipramine response, the idea of oestrogen administration was considered. Oestrogen was used, as oestrogens appear capable of producing mood changes of either sign in some women[82], therefore, although oestradiol was administered to thiry depressed women, so as to enhance the antidepressant activity of imipramine , Prange A.J. *et al*[83]. reported that five depressed patients on daily doses of 150 mg imipramine and 50 µg ethinyl oestradiol did not improve as much as ten women who received imipramine and a placebo after the first week of the drug trial. Also, the patients showed signs of toxicity. Four of the five

complained of severe lethargy and four of severe hypotension. Two had a coarse tremor and five showed signs of mild depersonalization.

The mechanism of imipramine-oestrogen interaction is unclear. Oestrogen may inhibit the N-oxidation and demethylation of imipramine causing accumulation of imipramine in the brain and other tissues, but this theory has not been critically evaluated. Other steroids such as norethindrone (a progesterone derivative) produced similar effects with imipramine in mice[84]. It has also been shown that ethinyl oestradiol inhibited the metabolism of ethyl morphine and hexobarbital in rats[75].

5.8.2 Imipramine - Methyl Testosterone in Men

Prange and coworkers[85] reasoned that if being 'more female' worsens the patient's response to imipramine (if only in the realms of toxicity), and if being male improves it as well as reducing the risk for depression, then being 'more male' might further improve the response to imipramine.

Five men were treated with 15 mg methyl testosterone and 50 mg imipramine. Four of the five patients given this treatment developed an acute paranoid reaction which began one to four days after medication treatment and resolved one to five days after methyl testosterone was stopped. All developed fearful delusions. In none of the five patients was there more than the usual degree of nuisance side effects that usually accompany imipramine treatment.

The cause of the paranoid syndrome is uncertain. Testosterone, like oestrogen is taken up by the brain discretely[86] and appears to enhance the activity of the noradrenergic system[87]. This might account for an enhanced antidepressant response although how it would account for paranoid reaction is uncertain.

It is suspected by Prange that the paranoid reaction is dose related. It seems possible that the use of smaller doses of methyl testosterone would avoid paranoid symptoms and enhance antidepressant response.

SECTION 6

MATERIALS AND METHODS

6.1 Metabolism Reactions Involving Isolated Rat Hepatocytes

Isolation and Use of Liver Cells

The technique described is based on liver perfusion with collagenase after removal of Ca^{2+} by preperfusion with a chelator. Moderately high yields of viable, single hepatocytes with a smooth spherical appearance and initially free of non-parenchymal cells were obtained.

The Test Animals

Albino female rats were used as the test animals. The animals were between 6 and 7 months old and weighed 200 ±20 g. Animals from a common source and stock were used since strain differences have been reported to be related to differences in drug metabolising enzymes[67]. Rats were allowed food and water *ad libitum* since Kato *et al*[88]. observed that starvation increased the metabolism of drugs.

Gas Mixture and Solutions

All solutions were bubbled with carbogen gas (95% O_2, 5% CO_2) and were heated to 37°C prior to use. The same gas mixture was used during perfusion of the liver and incubation of the hepatocytes.

Three different buffers were used consecutively. All chemicals used were of analytical grade.

HANK BUFFER CONCENTRATE

NaCℓ	80 g
KCℓ	4 g
$MgSO_4.7H_2O$	2 g
$Na_2HPO_4.2H_2O$	0,6 g
KH_2PO_4	0,6 g
Distilled H_2O to	1 litre

HANK SOLUTION

Hank Buffer Concentrate	25 mℓ
$NaHCO_3$	525 mg
Penicillin [Benzyl- 3 g (5×10^{-6} IE) in 100 mℓ]	2.5 mℓ
Distilled H_2O	222 mℓ
HEPES [Sigma No H 3375]	750 mg

FIRST PERFUSION FLUID - BUFFER A

Hank Solution	150 mℓ
Albumin (B.S.A. Sigma)	3 g
EGTA (Sigma E 3251)	34.2 mg

SECOND PERFUSION FLUID - BUFFER B

Hank Solution	100 mℓ
Collagenase	100 mg
Ca $C\ell_2.2H_2O$	58.8 mg

[Collagenase must be produced by Boehringer Mannheim from Clostridium histalyticum-lyphalized]

BUFFER C - KREBS-HENSELEIT BUFFER

H_2O		785 mℓ
$NaC\ell$	(16.09%)	200 mℓ
$KC\ell$	(1.10%)	150 mℓ
KH_2PO_4	(0.22M)	25 mℓ
$MgSO_4.7H_2O$	(2.74%)	50 mℓ
$CaC\ell_2.2H_2O$	(0.12M)	100 mℓ

Bubble with carbogen. Dissolve 9,71 g $NaHCO_3$ in 1 litre distilled water and bubble with carbogen. Mix the two solutions. Store in a dark flask in a refrigerator.

INCUBATION MEDIUM

Krebs Henseleit Buffer	200 mℓ
HEPES	0.75 g

All solutions should be at a pH 7.4 and could be adjusted with 1 M NaOH. Albumin lowers the pH.

TRYPAN BLUE SOLUTION

Trypan Blue	0.32 g
Krebs Henseleit Buffer	100.0 mℓ
Distilled H_2O	98.0 mℓ
HEPES	0.6 g
Albumin	4.0 g

Mix, and set aside stirring overnight. Filter. Place 0.99 mℓ in a test tube. Freeze for later use. Trypan Blue to be used must be microfine Flukon AG 93590.

Perfusion Apparatus

Figure 17 depicts the liver perfusion system. The buffer reservoir was a 6 cm wide glass beaker that was thermostatted to 37°C. A plastic rack consisting of two vertical tubes with a horizontal bar at the top and a tightly fitted screen with openings 1 x 1 mm wide in the bottom, was placed in the beaker. In the horizontal bar was a notch in which the cannula could be fixed. The oxygenator was a cylinder 10 cm high and 1.5 cm wide with four openings: one inlet for the gas mixture, one inlet for the perfusate, one outlet connected to the cannula (and the liver) and one outlet for the gas and shunted perfusate (bypassing the liver). The oxygenator also functioned as a bubble trap. The steel cannula had a smooth (filed) tip and a shallow (filed) groove around the cannula at 0.4 cm above

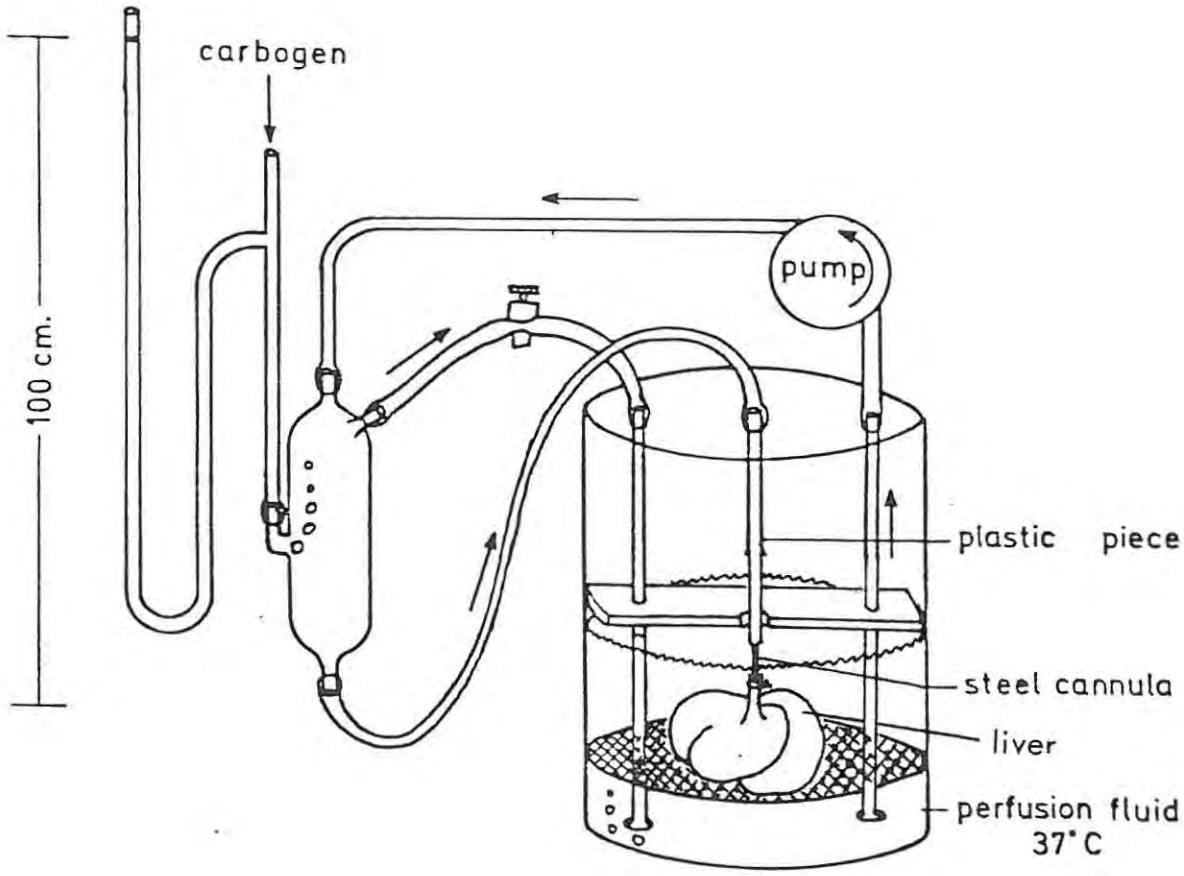


Fig. 17. LIVER PERFUSION SYSTEM

the tip to secure the cannula in the portal vein. The cannula permitted a flow of 100 mℓ/min with pressure used. The various parts were connected with Teflon tubing and the dead volume in the system (oxygenator and tubing) was 50 mℓ. The gas inlet of the oxygenator was connected to a gas cylinder with a pressure regulator with the centrifugal pump (Stuart) giving a constant flow rate (200 mℓ/min). The pressure in the oxygenator was dependent on the flow capacity of the shunt, which was regulated by the gas flow, and also by a screw clamp applied to the shunt; that is, the flow through the cannula could be varied from a few drops per minute to 100 mℓ/min by changing the gas flow.

Surgical Procedure

The rats were anaesthetized with ether and maintained. The peritoneal cavity was opened by a mid ventral incision. Heparin (500 units in 0.1 mℓ) was injected in the caval vein. A loose ligature was applied around the portal vein; an oblique incision was then made in the mesenteric part of the vein, and the cannula was immediately inserted. The cannula was first fixed with a clamp and then secured with the ligature. The perfusate flow rate was then adjusted to let the liver resume normal shape. The liver was excised by first removing the stomach and intestine in one piece, followed by liberation of the liver from the diaphagm, and, finally with the rat in a tilted position, by cutting the dorsal ligaments. The time from the insertion of the cannula until the liver had been freed was less than 2 minutes.

Perfusion and Washing Procedure

The perfusion was started *in situ* with buffer A. To avoid perfusing bubbles into the liver, the buffer was permitted to drip

out of the cannula before cannulation of the vein. A sign of adequate perfusion was that the liver cleared immediately and completely. When the liver had been freed from the body, it was immersed in the buffer in the reservoir and the cannula was fixed to a horizontal bar. After 4 minutes of perfusion with buffer A, after all the haemoglobin had been removed, the plastic rack with the liver was removed from the reservoir beaker and the oxygenator was almost emptied of perfusate by compressing the shunt. The rack was then placed in another beaker containing buffer B. Buffer B was recirculated for approximately 6 minutes. At the end of perfusion the liver appeared swollen and pale, but no blebs were seen on the surface. The liver was then immersed in 100 mℓ incubation medium (in a wide 150 mℓ capacity, low beaker), the capsula was cut open and the cells were dispersed with a pair of scissors by gentle stirring movements. Within 2 minutes, at ambient temperature, the dispersed cells were filtered through cotton gauze to remove connective tissue and clumps of cells. The filtrate was collected in a beaker 7 cm wide. Within 2-3 minutes at ambient temperature, the cells settled to form a loose pellet and the supernatant was removed by aspiration. The volume of the pellet (approximately 10 mℓ) was estimated with a pipette and the cells ($200-300 \times 10^6$) were counted in a haemocytometer chamber, using the white cell divisions.

Viability of Isolated Hepatocytes

Cell viability was routinely estimated by trypan blue staining prior to experiments.

Trypan Blue Exclusion Test

The number of viable cells in each batch was estimated by

counting a suspension that had been diluted 100 fold in trypan blue solution. Within 5 minutes 1-2% of the cells were stained. This indicated that more than 90% of the cells were still viable.

Incubation Procedures

Incubations were started immediately after isolation of the hepatocytes by diluting the sedimented cells in the incubation medium to final concentration of $1 - 3 \times 10^6$ cells/mℓ. This was done at ambient temperature, and the cells were never chilled below this temperature.

6.1.1 The Effect of Premarin^R on the Metabolism of Antidepressive Imipramine using Hepatocytes and HPLC as Detection Apparatus.

Controlled metabolic reactions were carried out using rat isolated hepatocytes. Reaction vessels allowed low concentration of imipramine with and without Premarin.^{R}

METHOD AND MATERIAL

Apparatus

High Performance Liquid Chromatography - Model Varian 8500

Absorbance Detector	Varian Aerograph ^{R} 254/280 nm UV Detector
Recorder	Varian Aerograph ^{R} CDS 111
Injector	Varian Aerograph ^{R} High Pressure Septumless Liquid Chromatography Injector

Column:- C_{18} reversed phase column 39 mm x 30 cm

μ Bondapak. Water Association

Additional equipment

50 mℓ Round bottomed flaskes

Buchi apparatus Model

250 mℓ centrifuge tubes (poly propylene)

15 mℓ conical pyrex centrifuge tubes

Reagents and Standards

All reagents were reagent grade (A.R.) unless otherwise specified.

Hexane UV grade

Methanol HPLC grade

Acetonitrile UV grade

Isoamyl alcohol

Pentane sulfonic acid (Pic B-5, 5 mmole/litre, water association)

Distilled water

Mobile phase - Methanol/acetonitrile/phosphate buffer (0,1 mol/litre pH 7,6) 41/15/44 by volume.

pH was adjusted to 6,5 by dropwise addition of concentrated sodium hydroxide or glacial acetic acid. The mixture was filtered through 0,45 µm membrane filter under negative pressure.

METHOD

a) Preparation of hepatocytes as described earlier.

b) Metabolic incubation.

50 mℓ round bottomed flasks were used. The order of additions was kept constant to prevent variations.

Reaction vessels	1	2	3	4
Imipramine HCℓ (5×10^{-2} M)	20 µℓ	20 µℓ	20 µℓ	20 µℓ
Premarin^{R} (5×10^{-2} M)	-	20 µℓ	-	20 µℓ
Hepatocytes (1×10^{6} cells/mℓ)	10 mℓ	10 mℓ	10 mℓ	10 mℓ

The final concentration of Premarin^{R} and imipramine was 1×10^{-4} M. This concentration was decided on as it was shown that antidepressives are toxic to rat hepatocytes at higher concentrations[89].

Incubation was allowed for 10 min at 37°C in round bottomed flasks fitted on a standard taper distillation adaptor for four flasks which was rotated (10 rpm) on a rotary evaporator (Buchi). The evaporator

was positioned so that axes of rotation deviated 45° from the water surface and so that the flasks dipped down in the thermostatted water. To ensure adequate oxygenation during the incubation, carbogen gas was applied continuously to the surface of the incubation medium through the central vacuum exit of the evaporator.

Enzymatic processes were stopped using 1 mℓ 10% trichloroacetic acid which precipitated the protein. Thereafter, extraction was performed. The liquid-liquid extraction methods are much simpler and more suitable for routine applications, as the recoveries of the tricyclic drugs were found by others to be highly variable when a standard extraction/evaporation/reconstitution procedure was used. The more manipulations the procedure involved, the less reproducible it was.

Reproducibility was acceptable when the tricyclic bases were simply extracted from alkalinized serum into hexane/isoamylalcohol (99/1) and re-extracted into a small volume of dilute hydrochloric acid. An aliquot of the aqueous acid was injected directly into the chromatograph, circumventing the evaporation step, which apparently was responsible for much of the observed variability in recoveries.

Use of relatively selective extract solvents combined with re-extraction of the basic amines into dilute acid removes most acidic and neutral interfering substances and leads to a sufficiently 'clean' extract that requires no further purification. It was found by others [90], that the solvent systems hexane/isoamylalcohol(99/1) presented the best compromise with respect to extraction efficiency and selectivity. Higher concentrations of isoamyl alcohol increased the background interference in the blank; lower proportions resulted in significant extraction losses of some of the tricyclic amines.

The concentration of sodium hydroxide used to alkalinize the serum sample was critical for optimium recovery; an excess decreased the recovery of the tricyclic amines.

Technique Used for Assay Procedure

Tricyclic drugs and their metabolites in serum are usually measured by gas liquid chromatography or high-performance liquid chromatography (HPLC). Gas liquid chromatography methods generally require a lengthy clean up procedure to eliminate interference from endogenous sample constituents, resulting in variable extraction losses and rendering them too cumbersome for routine clinical use[91,92].

In most published HPLC methods[89,93] liquid solid-chromatography (normal phase) on silica columns is used, with an aliphatic amine in the eluent to decrease chemisorption which causes peak tailing. This leads to early deterioration of column efficiency owing to gradual dissolution of the silica packing at pH value about 7. Also the predominantly hydrophilic endogenous plasma components and extraneous polar substances in the crude extracts have a high affinity for the polar packing materials, causing background interferences and necessitating long intervals for column recovery in between injections or heated column compartments[92]. For these reasons the use of reversed phase HPLC is preferred for clinical routine applications.

The technique used for this assay of tricyclic antidepressants and their metabolites is a modified method which uses a reversed phase column[94].

Extraction Procedure

1. 10 mℓ of serum was transferred into a 250 mℓ polypropylene centrifuge tube (50 mℓ tubes would have been preferred, however, no

head or tubes were available for MSE centrifuge that was used) and internal standard was added. The solution was alkalinized to pH 14 by adding sufficient volume of 1,5 mol/litre sodium hydroxide, and 10 mℓ hexane/isoamyl alcohol (99/1) was also added.

2. The solution was rotated and mixed for 5 minutes; gentle agitation prevented any emulsion forming during this step.

3. It was then centrifuged for 6 minutes at 500 x g (± 1800 rpm using MSE high speed centrifuge), and 9 mℓ of the organic (top) phase transferred into a 15 mℓ conical centrifuge tube.

4. 200 µℓ of 0,1 mol/litre HCℓ was added. The mixture was shaken for 10 minutes and centrifuged for 4 minutes at 500xg.

5. After discarding the organic phase, 10 µℓ of the aqueous acidic phase was injected into chromatograph using the following instrument settings.

Flow rate	90 mℓ/hr
Detector	UV 250 nm
Sensitivity	Perkin-Elmer Recorder - Range 10 and Varian CDS III - 8

The peaks that resulted were recorded on recorder paper. Substrate disappearance was measured and this was compared to internal standard, and a ratio of substrate height and internal standard height was measured and calculated. Metabolite formation was not measured, as in hepatocytes especially, further metabolism occurs, e.g. by various conjugations.

CHROMATOGRAPHIC CONDITIONS

Stationary Phase

To avoid the problems associated with normal phase silica columns in routine analysis of crude plasma extracts i.e. excessively

long retention of polar constituents, necessitating long column recovery times in between injections, a C-18 bonded reversed phase column was used.

Mobile Phase

Water/methanol and water/methanol/acetonitrile mixtures were tested as mobile phases in the pH range of 3-7 by others[90]. They found that while some of the drugs could be separated under these conditions, the selectivity factors were inadequate especially for separating the tertiary amines from their secondary bases. Also, the peak shapes of the longer retained compounds were not symmetrical, because of ionization of the amines in acidic and neutral solutions. Additions of a base (ammonia, ethylamine or propylamine) to elution solvent did improve the peak shape by supressing ionization but resolution was poor and column efficiency quickly deteriorated owing to channeling caused by gradual dissolution of the silica packing at the unfavourable pH.

To eliminate the problems associated with ionization of the tricyclic amines and to get their resolution at a pH below 7 to ensure reasonable column life, the paired-ion technique was used. Pentane sulfonate (Pic B-5 water association) was used as a counter ion.

Symmetrical peak shapes were obtained by using the solvent methanol/acetonitrile/phosphate buffer (41/15/44). The phosphate buffer was prepared with water that contained 5 mmol pentane sulfonate. The pentane sulfonate was prepared by diluting Pic B-5 associated waters with 1 litre of distilled water.

Effect of pH

A reported and actual finding was that the single most critical factor influencing the resolution of a tertiary and secondary amine

of identical fused-ring structure is the pH of the solvent system. At pH value <5 the tertiary compounds will not be separated from their secondary compounds. All the tricyclic antidepressive tertiary amines will be separated at pH 6,2 to 6,5. At higher pH the separation increases but also the peaks themselves which result are reduced in symmetry.

Detection in the Ultraviolet

The absorption maxima of tricyclic amines reported are about 235 nm for doxepin, 240 nm for nortriptyline, and amitriptyline, 250 nm for imipramine and desipramine, 229 nm for dothiepin, 252 nm for chlorimipramine and 250 nm for trimipramine.

RESULTS

Metabolism did occur, but Premarin[R] did not inhibit metabolism. Figs. 18, 19 show spectra where the internal standard was chlorimipramine HCℓ, and the effect Premarin[R] had on the substrate peak height. It can be clearly seen, that the presence of Premarin[R] did not significantly affect the substrate height.

CONCLUSION

Results were surprising, as reports have shown that in women taking conjugated oestrogens these have interacted with imipramine[95]. A possible explanation is that a lower affinity for the rat cytochrome P-450 for Premarin[R] may exist when comparing it to imipramine, since most of the oestrogens in Premarin[R] are conjugated.

A spectral study was done to see what effect Premarin[R] and ethinyl oestradiol would have on the K_s value for imipramine HCℓ

6.2 Spectral Techniques for Detection of Competitive Inhibition of Binding to Cytochrome P-450 between Two Substrates which elicit the Same Type of Spectral Changes

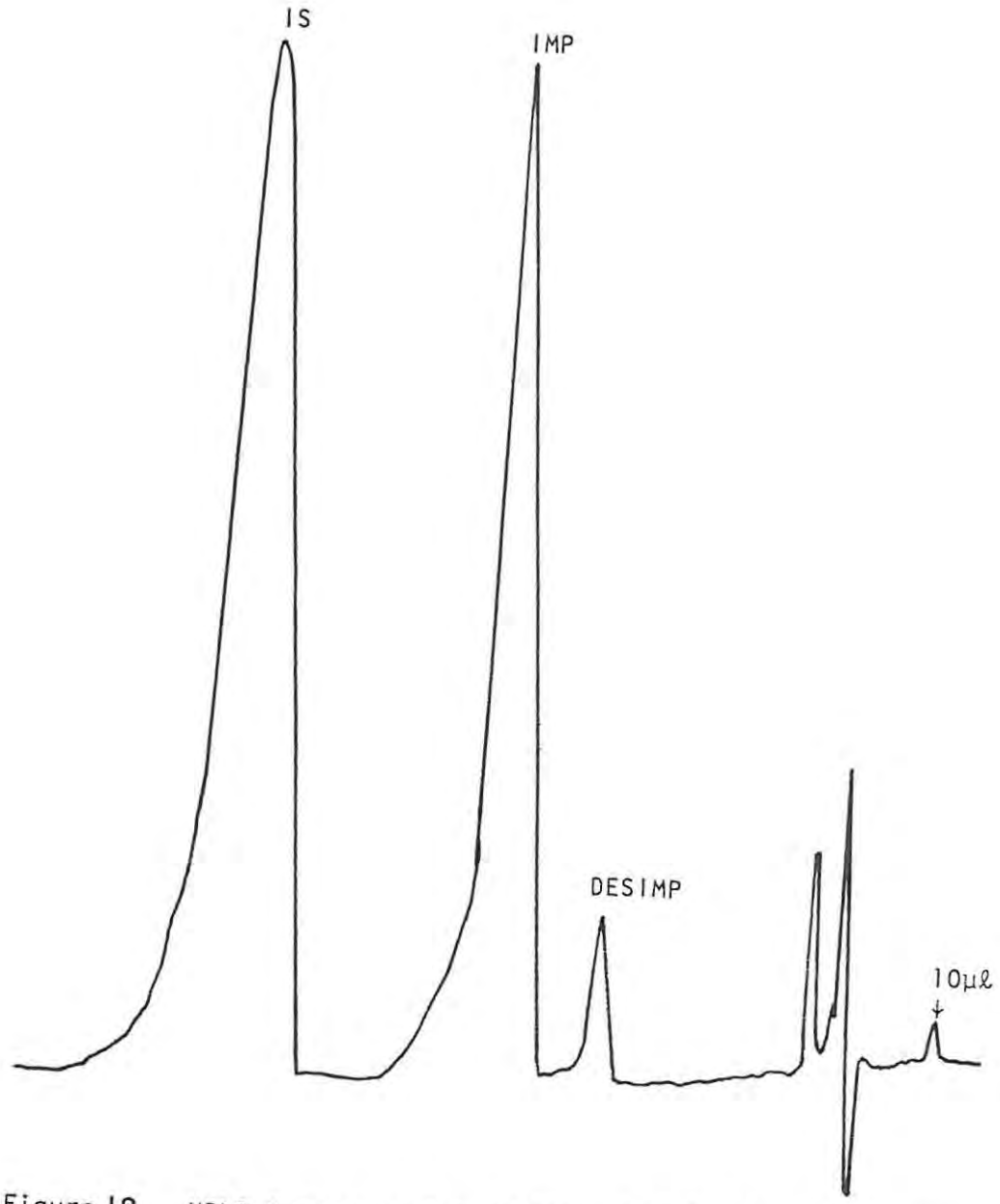


Figure 18. HPLC Spectrum of Imipramine HCℓ (IMP) Metabolised
Major Metabolite is Desimipramine (DESIMP)
Internal Standard (I.S.) Chlorimipramine HCℓ

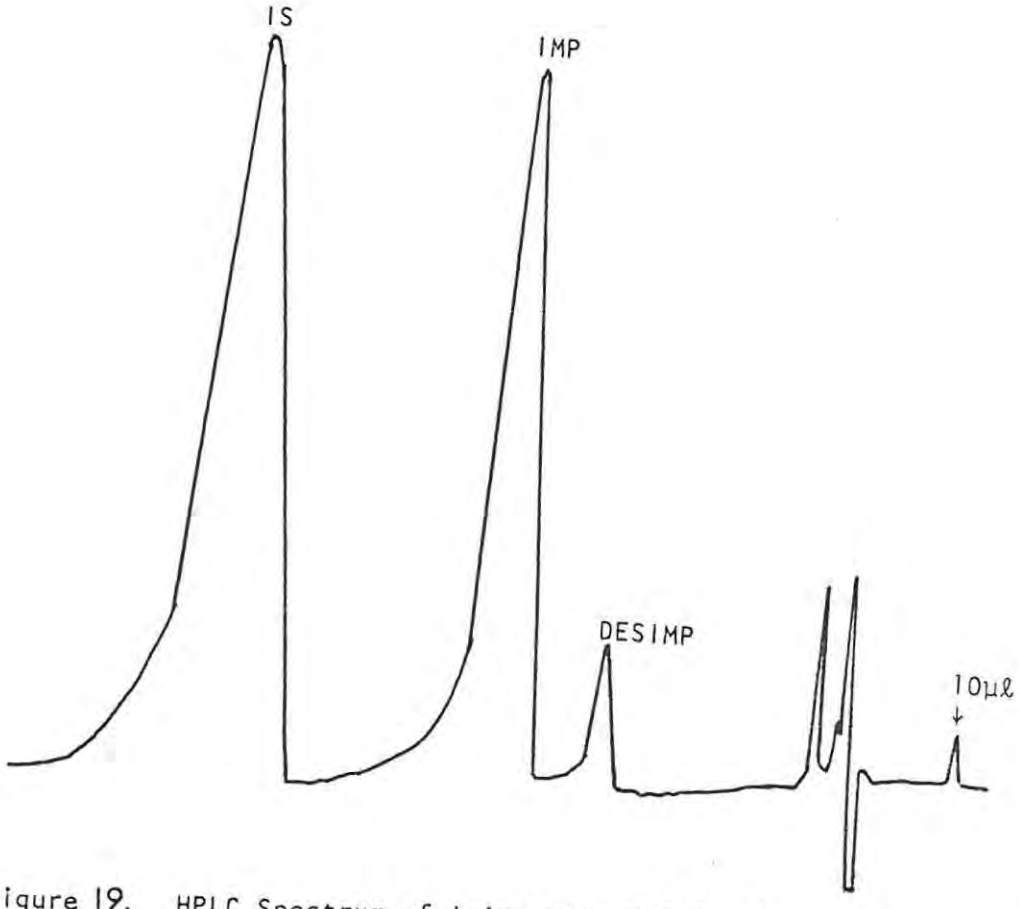


Figure 19. HPLC Spectrum of Imipramine HCℓ (IMP) Metabolised in the presence of Premarin^{R} (1×10^{-4} M)
Major Metabolite is Desimipramine (DESIMP)
Internal Standard (I.S.) Chlorimipramine HCℓ

This technique studies the spectral interaction with cytochrome P-450 and determines the K_s value. K_s values are again determined when saturable amounts of competitive inhibitor are present. An increase in K_s value will indicate that a competition for cytochrome P-450 does exist.

Microsomes were used instead of hepatocytes as special facilities were necessary to use hepatocytes, such as frequent stirring. The apparatus available could only accommodate microsomes.

PREPARATION OF RAT LIVER MICROSOMAL FRACTION

Method used was that of Cinte et al. (1972)[96].

Chemicals

Phosphate buffer, pH 7,4 prepared from KH_2PO_4 and $Na_2HPO_4.2H_2O$

0,25 M Sucrose

1,15% KCℓ

0,9% NaCℓ

$Ca.C\ell_2.2H_2O$

All reagents used were analytical reagents grade. All solutions were stored in a refrigerator.

All glassware used was thoroughly washed and rinsed in hot water, then soaked in chromic acid for 24 hours. After this, glassware was rinsed three times in distilled water and dried in an oven at 75°C. This cleaning procedure was necessary to ensure that the glassware was scrupulously clean to prevent contamination of the hepatic microsomal preparation.

Sacrificing the Animals

Animals were sacrificed by a blow on the head with a metal bar. To prevent circadian variation in hepatic microsomal activity, rats were sacrificed at the same time every morning between 09h00 to 09h15.

Once the animal had been sacrificed, the liver was quickly excised and weighed. Once the liver had been removed all operations were performed between 0°C-4°C, to prevent degradation of the enzymes. When the liver was removed, care was taken not to cut any of the lobes, since any damage would interfere with perfusion of the liver. Since haemoglobin would interfere with spectral examination of cytochrome P-450, it was removed from the liver by perfusion with 0,9% NaCℓ until the liver became lighter in colour[97]. A 25% w/v liver homogenate was always prepared (1g liver and 3 volumes sucrose); thus after perfusion the liver was placed into 3 volumes of cold 0,25 M sucrose solution and minced into small pieces with a clean pair of scissors.

Tissue Homogenization

After the liver had been minced, it was homogenized in a Thomas tissue homogenizer.

The steel rod of the teflon pestle was attached to an electric drill which allowed the teflon pestle to be rapidly rotated while in the glass homogenizer. The electric drill was connected to a rheostat, so that the speed could be controlled. To homogenize the tissue, the glass homogenizer tube was moved up and down relative to the pestle. This action and rapid rotation caused disintegration of the tissue and disruption of the cells, yielding a homogenate consisting of diluted cell sap, intracellular particles and some unbroken cells. These components were separated by fractional centrifugation.

The following precautions were observed during tissue homogenization. The liver was minced as finely as possible, since small pieces of tissue homogenize easier than large pieces, also to

prevent overheating of the tube and subsequent enzyme destruction.

The homogenizing tube was kept cold throughout the process by means of ice in a beaker, with the tube moving up and down in the cold water.

Centrifugation

The homogenate was transferred to two plastic centrifuge tubes and placed in a MSE 18 High Speed Centrifuge at 4°C.

The differential centrifugation was started at 4000xg ($5,8 \times 10^3$ revolutions per minute) for 10 minutes to allow all debris and nuclei to sediment, this was followed by 20 min at 10 000xg ($9,2 \times 10^3$ revolutions per minute) to allow mitochondria to sediment. Two steps were employed since fewer microsomes were trapped by sedimenting particles. The supernatant liquid was poured into a clean measuring cylinder and made up to 40 mℓ with cold 0,25 M sucrose. This liquid was then adjusted to a final concentration of 8 mM $CaCℓ_2$.

The Ca^{2+} makes aggregates of the endoplasmic reticulum membranes and thus causes the microsomes to sediment in 15-20 min at a force of 27000xg (15500 revolutions per min) [without Ca^{2+} one would have to apply a force of 105000xg for an hour]. The resulting microsomal pellet was washed by resuspending it in an equal volume of 1,15% KCℓ and resedimented at 27000xg. The new pellet was washed to remove excess Ca^{2+}, which may interfere with subsequent metabolic tests.

The final microsomal pellet was rehomogenized in phosphate buffer of pH 7,4 and the preparation appropriately diluted for studies.

Spectral studies were best done when the protein concentration was approximately 1 mg/1 mℓ.

Determination of Microsomal Protein.

Miller's Modified Lowry Protein Determination[98].

The coloured complex formed in this assay is only proportional to the protein concentration in the range of 50 to 200 µg/mℓ. Thus the microsomal suspension had to be diluted accordingly.

Method

B.S.A. of known water content was used as the standard protein and a calibration curve was constructed using standard solution of protein in place of the protein test solution. All determinations were performed in duplicate. 1 mℓ of the protein solution to be tested (aliquot protein solution was diluted a 100 fold) or 1 mℓ of water for the blank was added to 1 mℓ of the copper reagent, mixed and allowed to stand for 10 minutes at room temperature. 3 mℓ of diluted Folin-Phenol reagent was added and immediately mixed well. The tubes were heated for 10 minutes at 50°C in a water bath and the optical density was read at 450 nm. The values obtained from the calibration curve were corrected by the dilution factor. All reagents were freshly prepared from stock solutions.

Folin-Phenol reagent:- 5 mℓ of Folin-Phenol reagent was diluted with 50 mℓ of distilled water.

Copper Reagent:- (from the following stock solutions) 1 mℓ 1% copper sulphate; 1 mℓ 2% sodium tartrate; 20 mℓ 10% sodium carbonate in 0,5 M NaOH.

A Beckman DB 25 uv spectrophotometer was used to measure absorbance.

Results of Microsomal Protein Determination

Strength of solution (mg/mℓ)	0,05	0,1	0,15	0,2
Absorbance (nm)	0,65	1,22	1,695	2,17

Discussion

Miller's modified Lowry protein assay was used as workers like Albro[104] who investigated various methods for microsomal protein determination found it the most accurate.

The colour formed is thought to be due to a complex between the alkaline copper phenol reagent and the tyrosine and tryptophan residues of the protein.

6.2.1 The Determination of Spectral Dissociation Constant (Ks) for Imipramine Hydrochloride and the Influence that Saturable Amounts of Premarin^{R} and Ethinyl Oestradiol has on the Imipramine Ks Value

A method is described which allows

a) The determination of compounds used, binding to give a type 1 spectrum.

b) The estimation of dissociation constant for type 1 binding.

c) The detection of competitive inhibition of binding to cytochrome P-450 between two substrates which elicit the same type of spectral change.

The simultaneous binding of two compounds to the same site on cytochrome P-450 may be examined, provided that both compounds show a type 1 binding spectrum. The spectral effect observed on simultaneous additions to microsomal suspension cannot be separated.

Materials and Methods

The biochemical methods, such as preparation of liver microsomes, have already been described.

A Beckman Acta MVI ultraviolet spectrophotometer was set up as detailed in the operating booklet.

Wavelength scanned 500-360 nm (Tungsten source)

Period selector switch 2

Span control 0.02

Mode switch set at DB. Serve slit position

Slit program selector set on program position

A. Determination of Cytochrome P-450

Reagents

Prepared hepatic microsomal suspension

Carbon monoxide

$Na_2S_2O_4$

Method

Carbon monoxide difference spectra of cytochrome P-450 were run on a Beckman Acta MVI Spectrophotometer in the range from 500 nm to 360 nm.

All procedures were carried out at room temperature.

1. 2 mℓ of microsomal suspension was placed into each of two matched cuvettes of 1 cm optical path length.
2. A baseline was prepared with the aid of a set of potential adjustments every 20 nm from 500 nm to 360 nm.
3. The microsomal suspension from each cuvette was pooled and the solution reduced with a few milligrams of fresh $Na_2S_2O_4$, since exposure of powdered $Na_2S_2O_4$ to air and moisture results in its decomposition to an inactive form. If a strong pungent odour of bisulfide is apparent the sample of $Na_2S_2O_4$ should be discarded.
4. 2 mℓ was pipetted out into the sample cell and the remaining 2 mℓ returned to the reference cuvette.
5. The sample cuvette was then treated with carbon monoxide by

bubbling the gas through for one minute.

6. The spectrum was then recorded from 500 nm to 360 nm.

7. Each spectrum was then traced out and suitable adjustments for irregularities in the baseline effected.

8. The amount of cytochrome P-450 can be calculated from optical difference (450-490 nm) and a molar extention coefficient of 91 nM^{-1} cm^{-1} [99] .

B. 2 mℓ of the microsomal suspension was pipetted into precisely matched cuvettes i.e. identical light path, and placed in the sample and reference compartments of the scatter transmission section of the instrument. The cuvettes were placed in the optical path with their frosted rather than their clear faces in the light beams. This ensures a more uniform scattering of light through the sample thereby giving a better statistical representation of the contents of the cuvettes[97] . It was found that dilute microsomal suspension (0,5-1,0 mg/mℓ) gave a more satisfactory baseline. A baseline was prepared with the aid of a set of potential adjustments every 20 nm from 500 nm to 360 nm.

The spectral changes were produced by titrating small amounts of imipramine hydrochloride using a Hamilton micro litre syringe. Where large volume additions were involved an equal volume of distilled water was added to the reference cuvette. The spectrum was then recorded from 500 nm to 360 nm. A number of spectra for imipramine were performed so as to establish a Ks value (Ks is defined as the concentration of reactant that results in a spectral change of 50% of the theoretical maximal spectral change obtainable).

Thereafter, further Ks values for imipramine were determined.

However, in these experiments saturating concentrations of Premarin^{R} or ethinyl oestradiol were present which had been determined spectrally previously. The reference cuvette had also been saturated and on a straight baseline generated before addition of imipramine to the sample cuvette. If competition for the same cytochrome P-450 binding site existed, then an increase in the Ks value would result.

Each spectrum was traced out and suitable adjustments for irregularities in the baseline effected. Ks values were determined by the double reciprocal plot of spectral changes ($^{1}/\Delta A$) vs substrate concentration ($^{1}/[S]$).

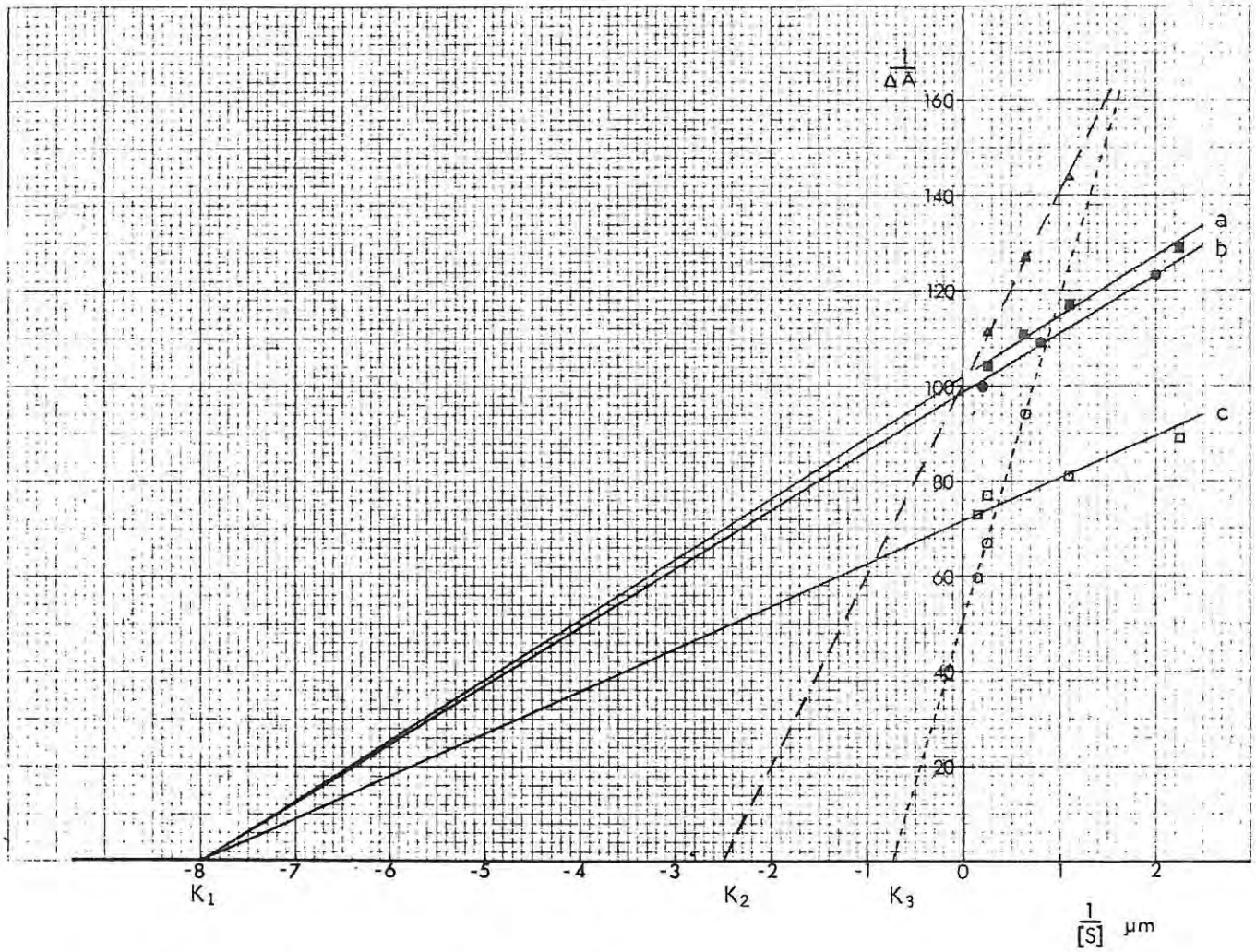
The cytochrome P-450 levels were also determined for each batch of microsomes used. This was done as spectral changes resulting from substrate interaction with cytochrome P-450 are dependent not only on the concentration of substrate but also on the concentration of cytochrome P-450 in the microsomal suspension .

Results

Imipramine hydrochloride, Premarin^{R} and ethinyl oestradiol all showed type I spectra. The Ks value for imipramine hydrochloride determined after three determinations was $0,125 \times 10^{-6}$ M. This value compared favourably with that determined by von Bohr et al. which was $0,22 \times 10^{-6}$ M.

Figure 20 shows the shift of Ks value with the presence of saturable amounts of Premarin^{R} and ethinyl oestradiol. It also shows the effect that varying concentrations of cytochrome P-450 have. Concentration is expressed as mμ moles.

The results show that ethinyl oestradiol caused a greater shift, resulting in a high Ks value for imipramine hydrochloride. This would indicate more imipramine hydochloride was required to obtain the same spectra as compared to when Premarin^{R} was used.



– – – – – Saturable amounts of Premarin^R

--------- Saturable amounts of ethinyl oestradiol

a = Resulting graph when concentration of cytochrome P450 is 0,565 mµmoles

b = Resulting graph when concentration of cytochrome P450 is 0,702 mµmoles

c = Resulting graph when concentration of cytochrome P450 is 0,846 mµmoles

K_1 = Binding constant of imipramine hydrochloride = $\frac{1}{[8]}$ µm = $0,125 \times 10^{-6}$ M

K_2 = Binding constant of imipramine hydrochloride in the presence of saturable amounts of Premarin^R = $\frac{1}{[2,5]}$ µm = $0,4 \times 10^{-6}$ M

K_3 = Binding constant of imipramine hydrochloride in the presence of saturable amounts of ethinyl oestradiol = $\frac{1}{[0,75]}$ µm = $1,33 \times 10^{-6}$ M.

Fig. 20 Graphic Determination of Spectral Binding Constant K_S of Imipramine Hydrochloride.

Conclusion

This method used was only a comparative technique to determine which of the two inhibitors would cause a greater Ks shift for imipramine hydrochloride. A much smaller shift when Premarin[R] was used may account for the reason why Premarin[R] did not significantly inhibit imipramine hydrochloride metabolism when hepatocytes were used.

The lower value when Premarin[R] was used indicates that Premarin[R] did not bind as strongly as ethinyl oestradiol to cytochrome P-450. Based on this observation, one could conclude that ethinyl oestradiol may be a stronger metabolic inhibitor than Premarin.[R] On this assumption metabolic reactions were carried out, however, this time microsomes instead of hepatocytes were used, because of the simplicity of obtaining microsomes as compared to hepatocytes, therefore lending itself to a more rapid laboratory procedure.

6.3 Determination of the effect of Ethinyl Oestradiol on the Metabolism of Tricyclic Antidepressives

Controlled metabolic reactions were carried our using rat liver microsomes. Reaction vessels contained varying concentrations of ethinyl oestradiol and the extent of metabolism of the imipramine in each was determined.

Methods and Material

Materials used have been described already.

Method

a) The microsomes were prepared as described earlier.

b) Metabolic Incubation

50 mℓ round bottomed flasks were used. The following compounds were added to each flask and the order of additions was kept constant to prevent variations.

The following solutions were prepared:-

5 mM $MgCl_2$ and 0,005 mM $MnCl_2$/0,2 mℓ

NADP 1,4 mg/Isocitric Acid 2,5 mg/0,2 mℓ

Antidepressives 1×10^{-2} M

Ethinyl Oestradiol 1×10^{-2} M, 2×10^{-3} M.

Reaction Vessels

		1	2	3	4
1.	Mn^{++}/Mg^{++}	200 µℓ	200 µℓ	200 µℓ	200 µℓ
2.	Distilled Water	ad qs 2,0 mℓ			
3.	NADP/Isocitric Acid	200 µℓ	200 µℓ	200 µℓ	200 µℓ
4.	Antidepressives	20 µℓ	20 µℓ	20 µℓ	20 µℓ
5.	Ethinyl Oestradiol				
	a) 1×10^{-2} M	-	200 µℓ	-	-
	b) 2×10^{-3} M	-	-	300 µℓ	-
	c) 2×10^{-3} M	-	-	-	100 µℓ
6.	Microsomes	1,0 mℓ	1,0 mℓ	1,0 mℓ	1,0 mℓ
7.	Isocitrate Dehydrogenase	20 µℓ	20 µℓ	20 µℓ	20 µℓ

The final concentration of antidepressives in reaction vessels was 1×10^{-4} M.

The final concentration of ethinyl oestradiol was in

vessel 2 = 1×10^{-3} M

vessel 3 = 3×10^{-4} M

vessel 4 = 1×10^{-4} M

The NADPH generating system functions in the following way :-

$$\text{D.L Isocitric Acid} + \text{NADP} \xrightarrow[\text{Isocitrate Dehydrogenase}]{Mg^{++}\ /\ Mn^{++}} \text{a Ketoglutaric Acid} + H^+ + CO_2 + \text{NADPH}$$

Incubation was allowed for 10 minutes at 37°C in round bottomed flasks fitted onto a standard distillation adaptor for four flasks which was rotated at 10 rpm on a rotary evaporator (Buchi). The evaporator was positioned so that the axis of rotation deviated 45°C from the water surface, so that the flask dipped down into the thermostatted water. To ensure adequate oxygenation during the incubation, carbogen gas was applied continuously to the surface of the incubation medium through the central vacuum exit of the evaporator.

The enzymatic process was stopped using 1 mℓ 10% trichloroacetic acid, which precipitated the protein. The respective internal standards were then added.

Choice of Internal Standard for HPLC

An internal standard must be similar in structure to the compound being studied. In this case a different tricyclic antidepressive was used, than the one under investigation.

Before any tricyclic antidepressive could be used as the internal standard, it was necessary to run a metabolic reaction. The spectra were recorded using HPLC. The spectra influence the choice, as an internal standard must not appear where other peaks are present. Also the standard should be eluted quickly, so as to reduce the time between each run. Another factor to be considered is the concentration of internal standard to be used. It was found that the tricyclic antidepressives varied as far as extraction efficiency was concerned. Therefore concentrations were determined experimentally so that all the peaks fell in the required scale. The table shows which internal standards were used and concentrations used.

	Internal Std.	Concⁿ.	Volume used
Imipramine HCℓ	Chlorimipramine HCℓ	0,1 g/10 M	10 µℓ/2 mℓ
Amitriptyline HCℓ	Doxepin HCℓ	1×10^{-2} M	10 µℓ/2 mℓ
Chlorimipramine HCℓ	Doxepin HCℓ	1×10^{-2} M	10 µℓ/2 mℓ
Trimipramine Maleate	Doxepin HCℓ	1×10^{-2} M	10 µℓ/2 mℓ
Doxepin HCℓ	Amitriptyline HCℓ	1×10^{-2} M	40 µℓ/2 mℓ
Dothiepin HCℓ	Amitriptyline HCℓ	1×10^{-2} M	40 µℓ/2 mℓ

Extraction Procedure

1. 2 mℓ of serum was transferred into 250 mℓ polypropylene centrifuge tubes. The solution was alkalinized to pH 14 by adding 600 µℓ 5,0 mol/litre sodium hydroxide, and 10 mℓ hexane/isoamyl alcohol (99/1) was also added
2. This was rotated and mixed for 5 minutes; gentle rotation prevented any emulsion forming during this step.
3. It was centrifuged for 6 minutes at 500 xg and 9 mℓ of the organic (top) phase was transferred into a 15 mℓ conical centrifuge tube.
4. 200 µℓ of 0,1 mol/litre HCℓ was added. The mixture was shaken for 10 minutes and centrifuged for 4 min at 500 xg.
5. After discarding the organic phase the aqueous acidic phase was injected into HPLC chromatograph . The injection volumes did vary, the following volumes were used:- Imipramine HCℓ, Amitryptyline HCℓ and Doxepin HCℓ and Dothiepin HCℓ, injection volumes were 10 mℓ. Trimipramine maleate and chlorimipramine HCℓ injection volumes were 50 mℓ. Larger Volumes of trimipramine maleate and chlorimipramine HCℓ were used because the extraction efficiency of these compounds is lower.

Instrument Settings

Flow rate	90 mℓ/hr	
Sensitivity	Perkin-Elmer Recorder - Range 10 and Varian CDS III - 8	
Detector	uv 250 nm filter	Imipramine HCℓ
	240 nm	Amitriptyline HCℓ
	252 nm	Chlorimipramine HCℓ
	250 nm	Trimipramine maleate
	235 nm	Doxepin HCℓ
	229 nm	Dothiepin HCℓ

The peaks that resulted were recorded on recorder paper. Substrate disappearance was measured. This was compared to internal standard and a ratio of substrate height and internal standard heights was measured and calculated.

Chromatographic conditions were the same as for hepotocytes, as covered in section 5.1.

Results

Figures 21a, 21b, 21c, 21d, 22a, 22b, 22c, 22d, 23a, 23b, 23c, 23d, 24a, 24b, 24c, 24d, 25a, 25b, 25c, 25d, 26a, 26b, 26c, 26d, show representative spectra of the tricyclic antidepressives under study, with their varying internal standards. The spectra show the influence that varying concentrations of ethinyl oestradiol had on the metabolism of the tricyclic antidepressives. The metabolites were labelled in cases where they could be identified.

From the spectra, substrate peak heights and internal standard peak heights were measured. From these measurements, the ratios of substrate peak heights to internal standard peak heights were calculated and

tabulated as shown in Table 6 . Three metabolic reactions were performed. The ratios were then expressed as a percentage metabolic activity, where the smallest ratio represented maximal metabolism. Using these values, a graphical representation was made as shown in Figure 27. The various graphs clearly show that as the concentration of ethinyl oestradiol increased the metabolic activity of rat microsomes decreased.

Conclusion

From these results it showed clearly that ethinyl oestradiol strongly inhibited the metabolism of various tricyclic antidepressives and this supports the concept put forward earlier that ethinyl oestradiol would be a stronger metabolic inhibitor than Premarin^{R}.

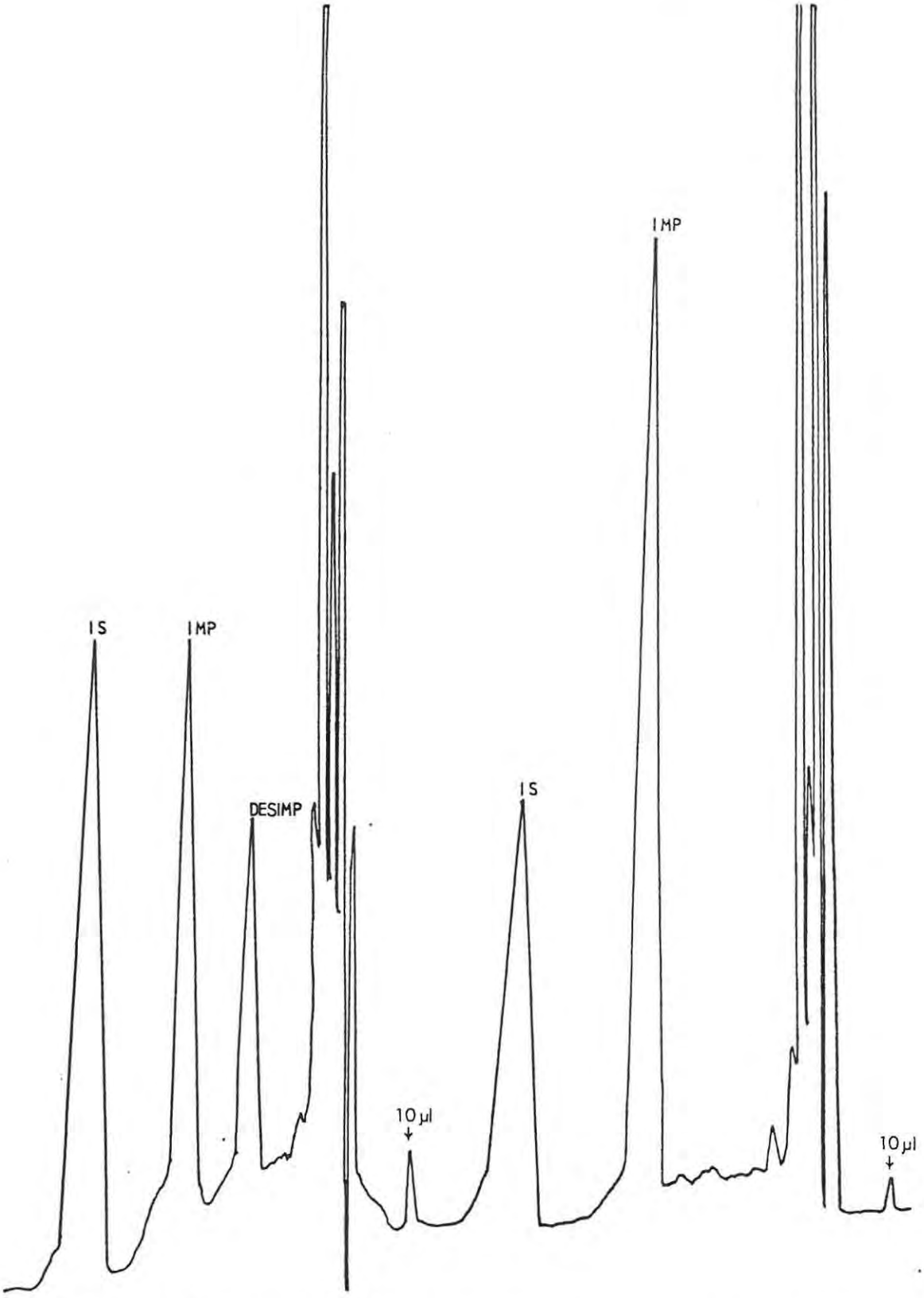


Figure 21a HPLC Spectrum of Imipramine HCℓ (IMP) Metabolised
Major Metabolite Desimipramine (DESIMP)
Internal Standard (I.S.) Chlorimipramine HCℓ

Figure 21b HPLC Spectrum of Imipramine HCℓ (IMP) Metabolised in the presence of Ethinyl Oestradiol (1×10^{-3} M)
Internal Standard (I.S.) Chlorimipramine HCℓ

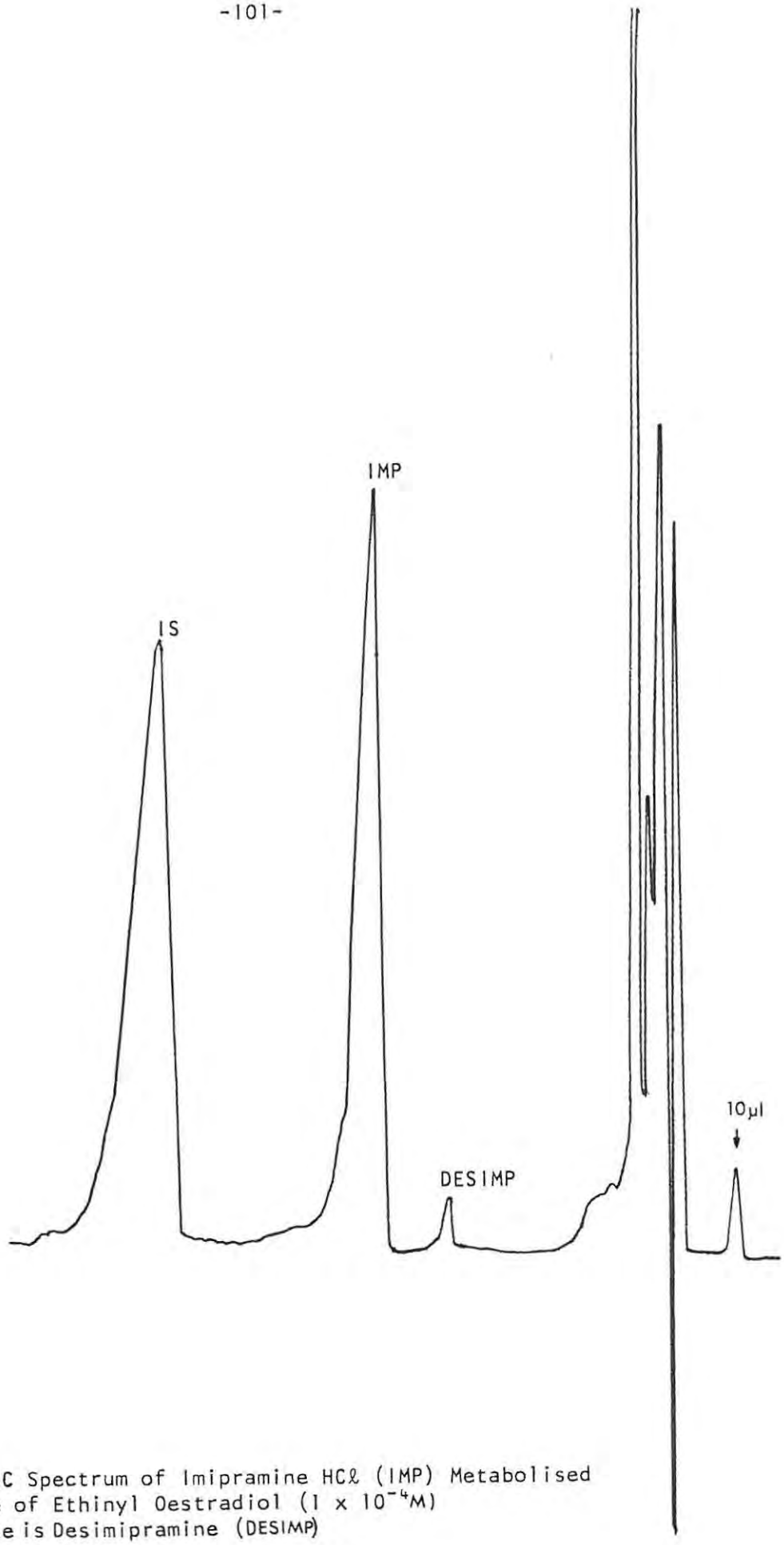


Figure 21c HPLC Spectrum of Imipramine HCℓ (IMP) Metabolised in the presence of Ethinyl Oestradiol (1×10^{-4}M)
Major Metabolite is Desimipramine (DESIMP)

Internal Standard (I.S.) Chlorimpramine HCℓ

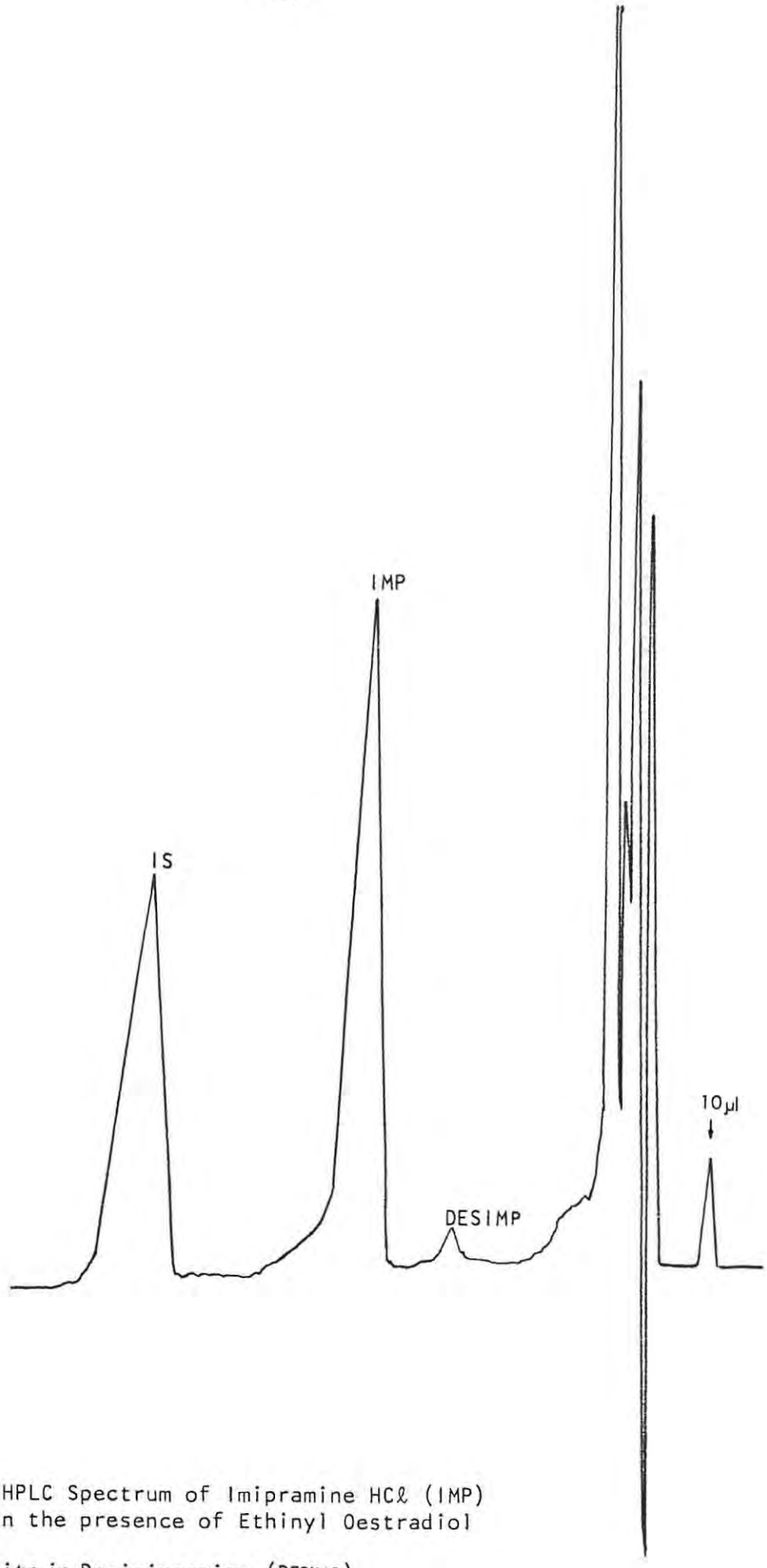


Figure 21d HPLC Spectrum of Imipramine HCℓ (IMP) Metabolised in the presence of Ethinyl Oestradiol (3×10^{-4} M)
Major Metabolite is Desimipramine (DESIMP)
Internal Standard (I.S.) Chlorimipramine HCℓ

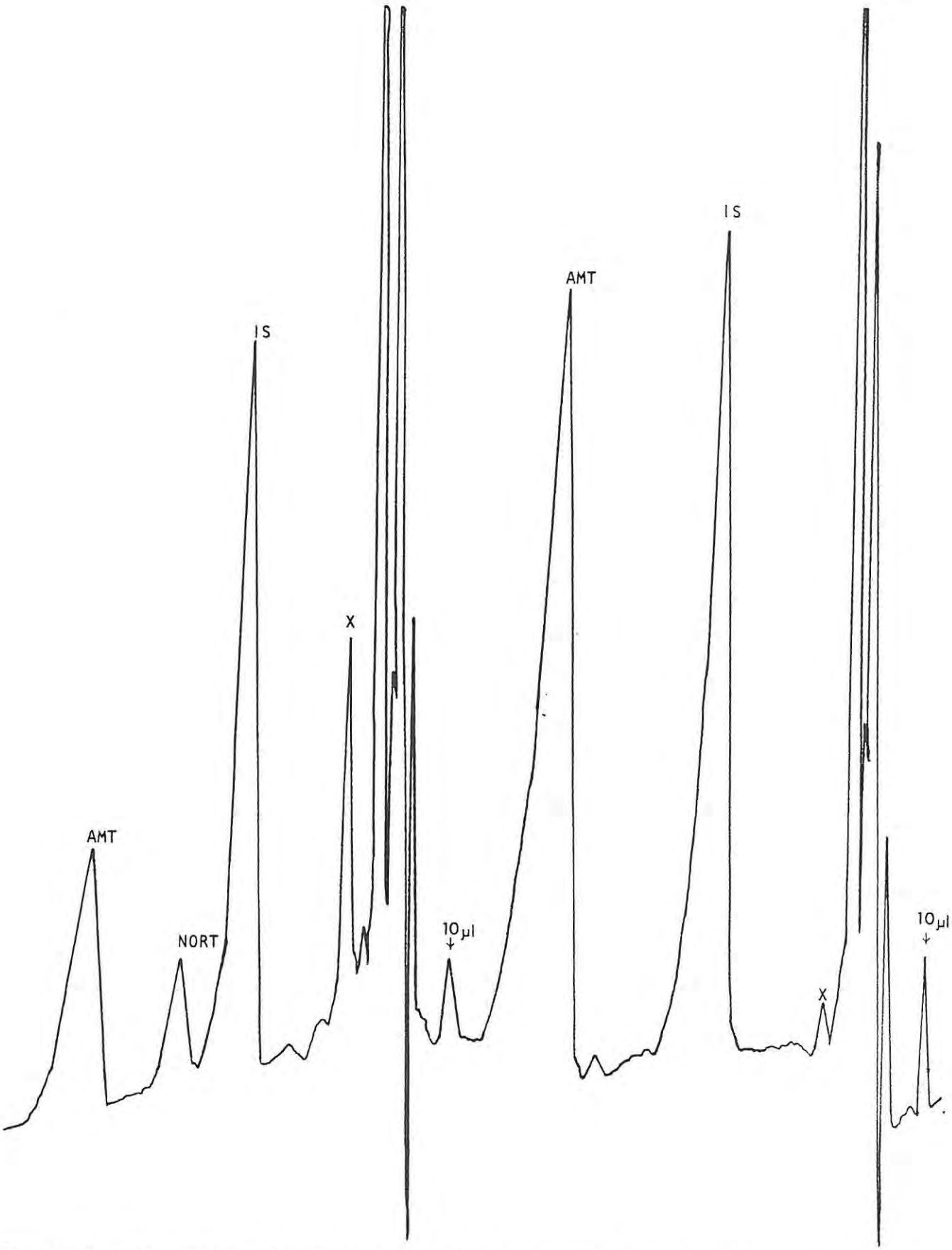


Figure 22a HPLC Spectrum of Amitriptyline HCℓ (AMT) Metabolised
Major Metabolite is Nortriptyline (NORT)
Internal Standard (I.S.) Doxepin HCℓ

Figure 22b HPLC Spectrum of Amitriptyline HCℓ (AMT) Metabolised in the presence of Ethinyl Oestradiol (1×10^{-3} M)
Internal Standard (I.S.) Doxepin HCℓ

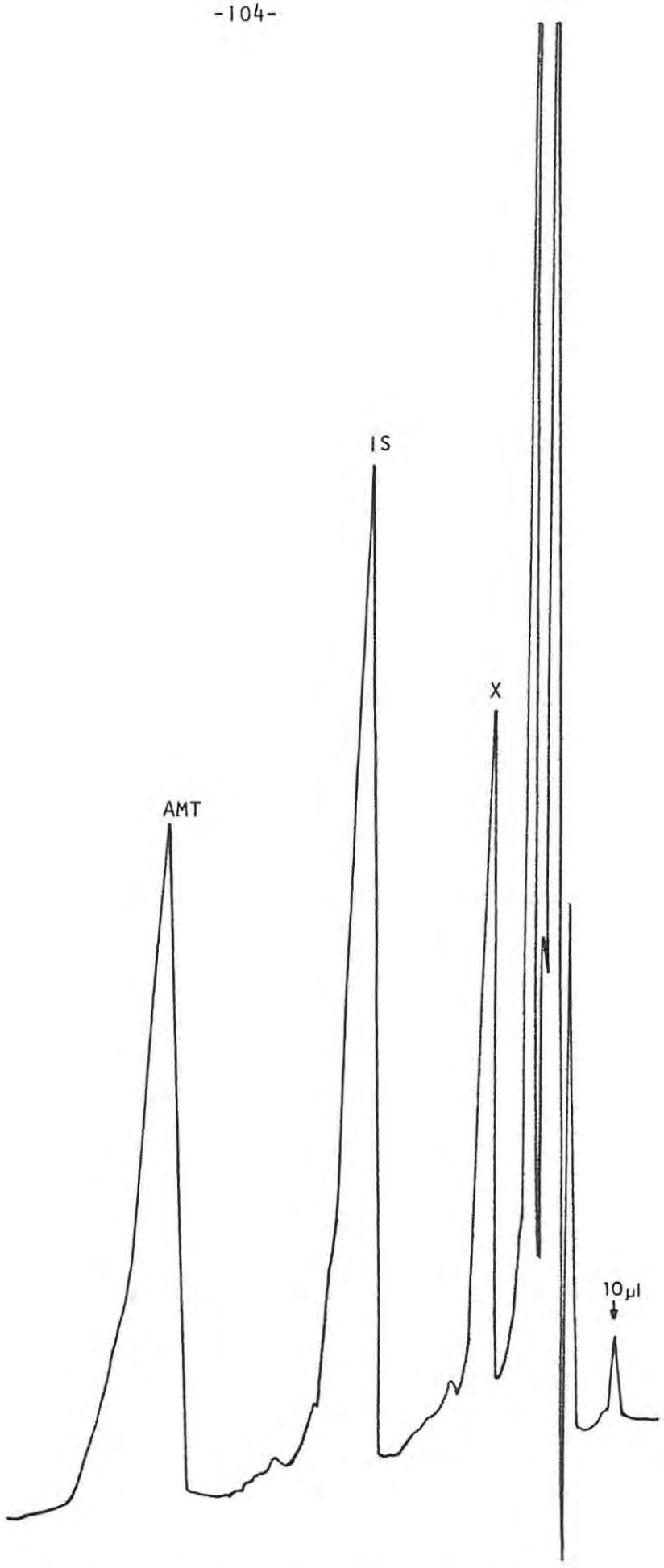


Figure 22c HPLC Spectrum of Amitriptyline HCℓ (AMT) Metabolised in the presence of Ethinyl Oestradiol (1×10^{-4} M)

Internal Standard (I.S.) Doxepin HCℓ

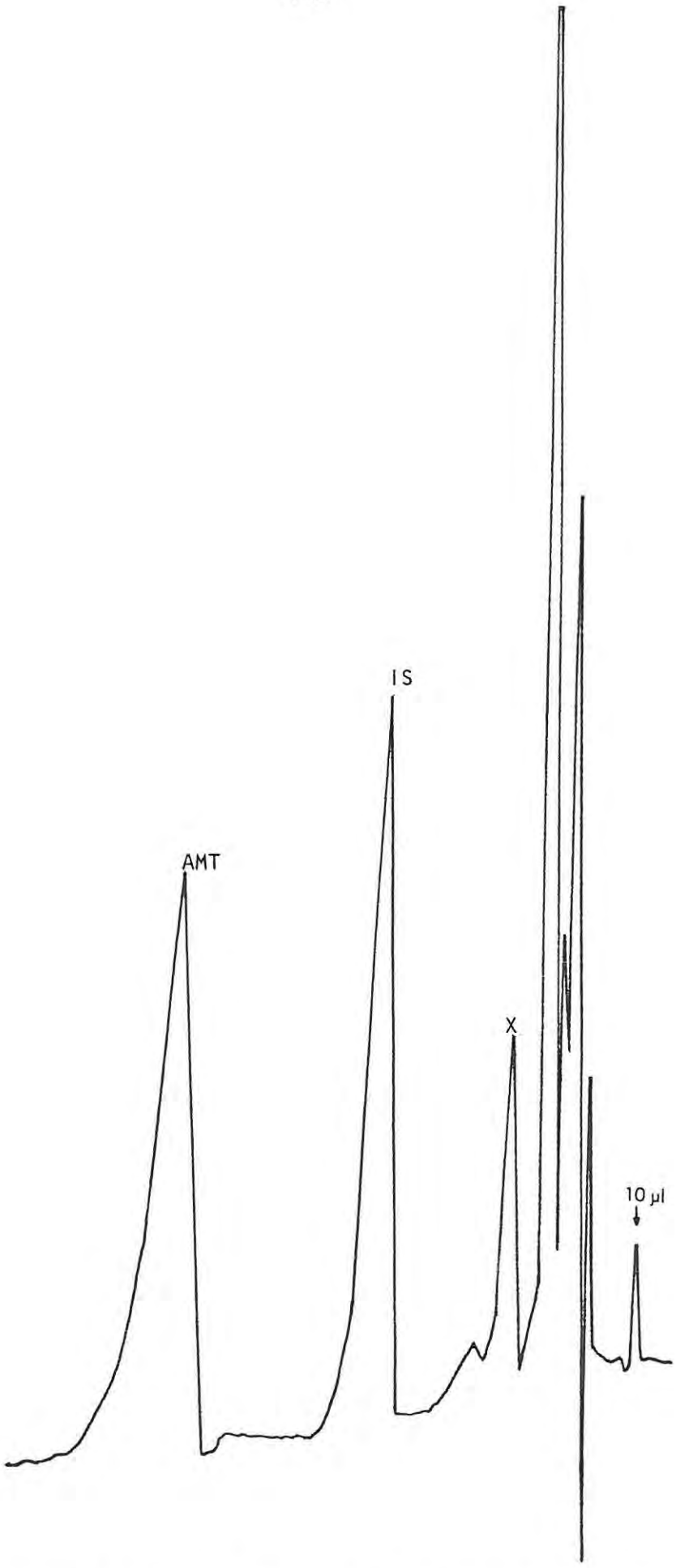


Figure 22d HPLC Spectrum of Amitriptyline HCℓ (AMT) Metabolised in the presence of Ethinyl Oestradiol (3×10^{-4} M)

Internal Standard (I.S.) Doxepin HCℓ

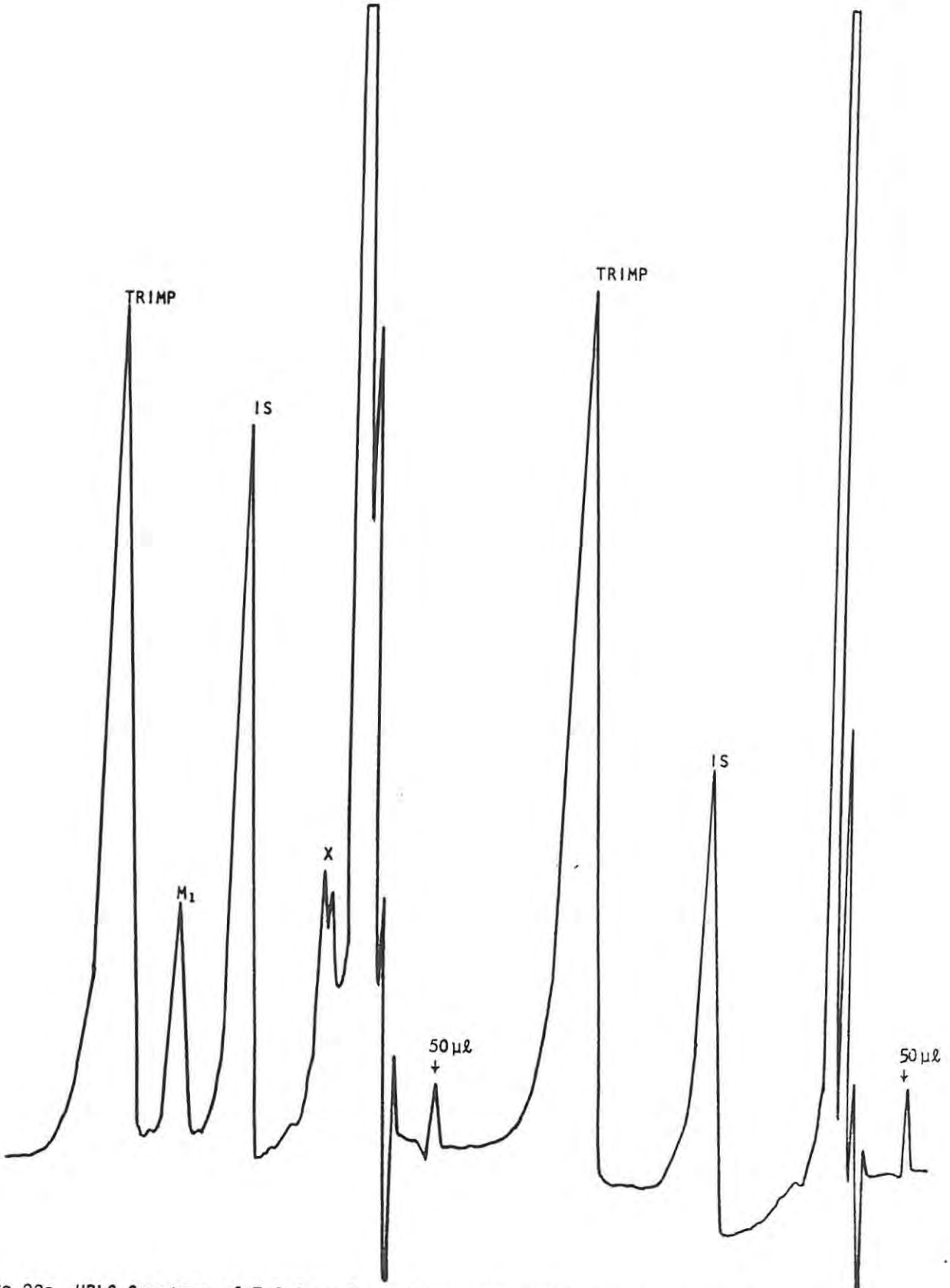


Figure 23a HPLC Spectrum of Trimipramine Maleate (TRIMP) Metabolised

Internal Standard (I.S.) Doxepin HCℓ

Figure 23b HPLC Spectrum of Trimipramine Maleate (TRIMP) Metabolised in the presence of Ethinyl Oestradiol (1×10^{-3} M)

Internal Standard (I.S.) Doxepin HCℓ

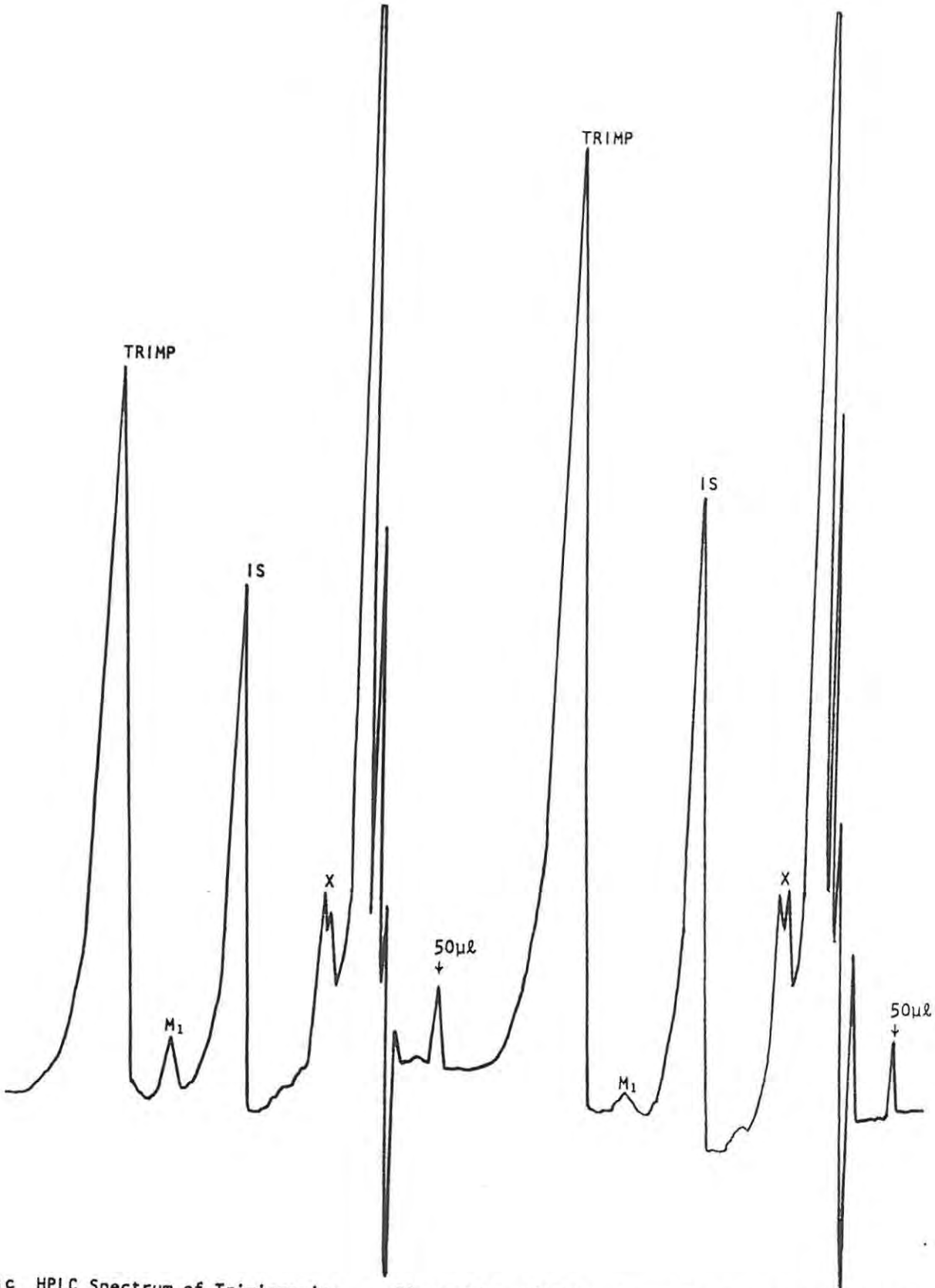


Figure 23c HPLC Spectrum of Trimipramine Maleate (TRIMP) Metabolised in the presence of Ethinyl Oestradiol (1×10^{-4} M)

Internal Standard (I.S.) Doxepin HCℓ

Figure 23d HPLC Spectrum of Trimipramine Maleate (TRIMP) Metabolised in the presence of Ethinyl Oestradiol (3×10^{-4} M)

Internal Standard (I.S.) Doxepin HCℓ

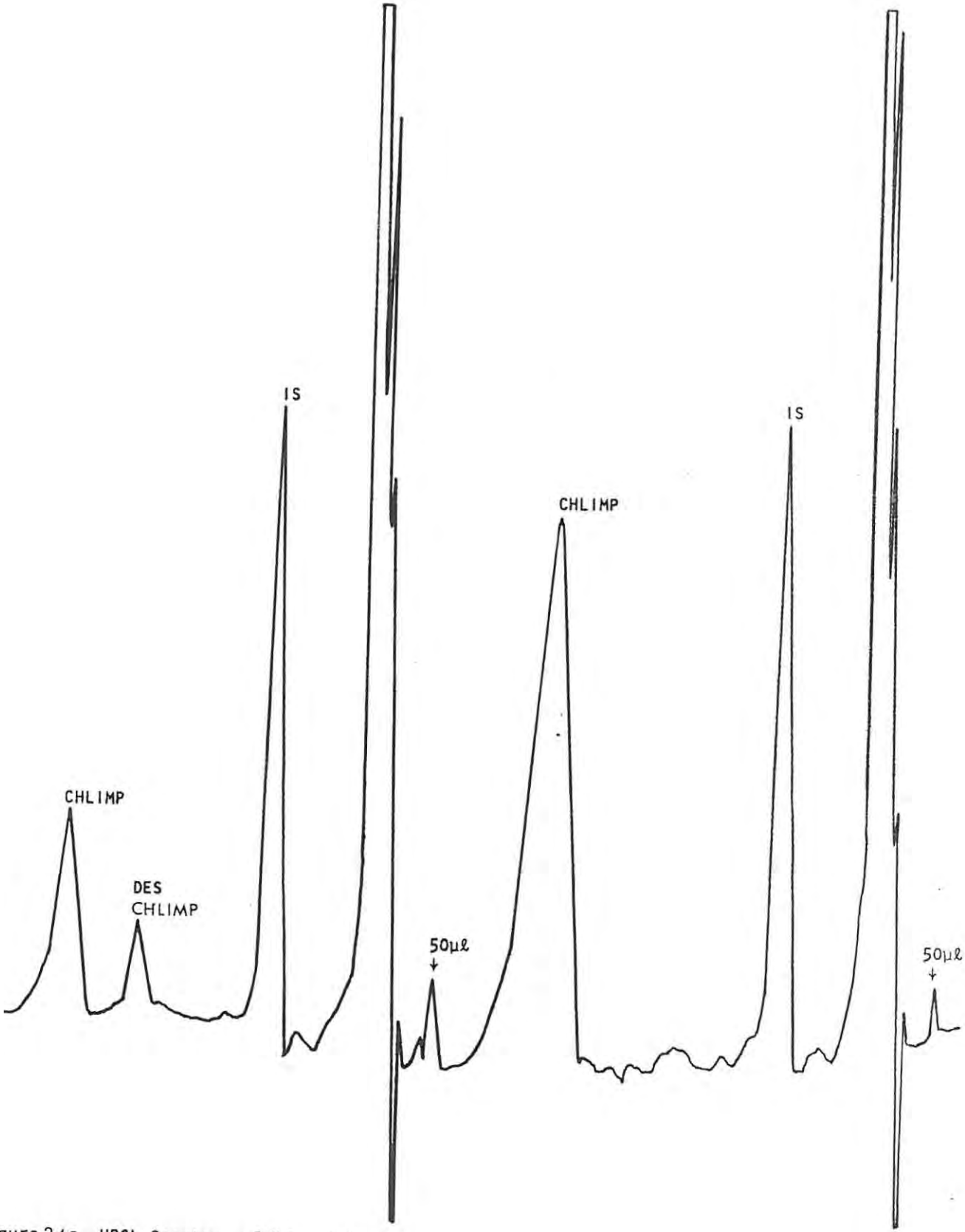


Figure 24a HPCL Spectrum of Chlor-imipramine HCℓ (CHLIMP) Metabolised

Internal Standard (I.S.) Doxepin HCℓ

Figure 24b HPLC Spectrum of Chlor-imipramine HCℓ (CHLIMP) Metabolised in the presence of Ethinyl Oestradiol (1×10^{-3} M)

Internal Standard (I.S.) Doxepin HCℓ

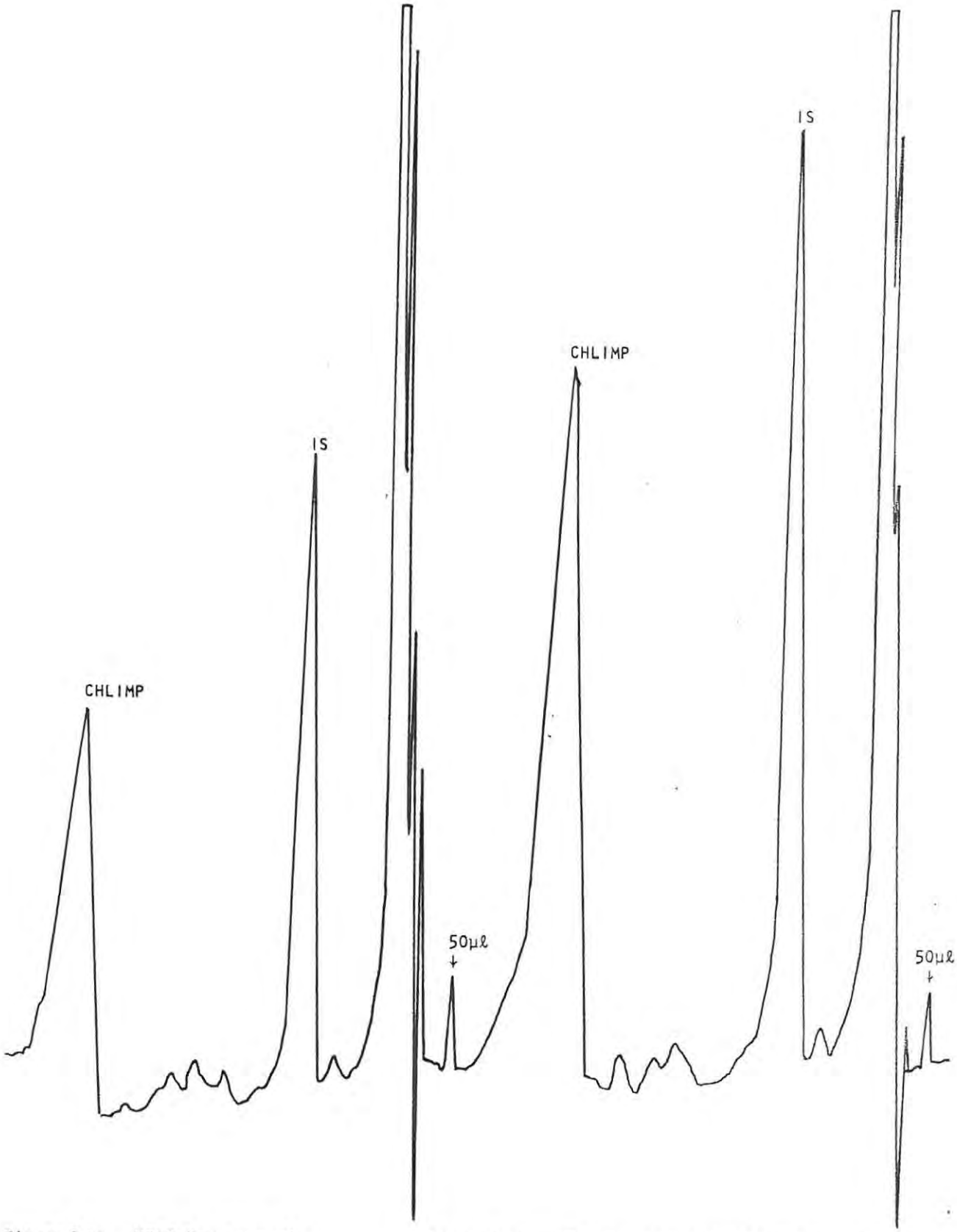


Figure 24c HPLC Spectrum of Chlorimipramine HCℓ (CHLIMP) metabolised in the presence of Ethinyl Oestradiol (1×10^{-4})
Internal Standard (IS) Doxepin HCℓ

Figure 24d HPLC Spectrum of Chlorimipramine HCℓ (CHLIMP) metabolised in the presence of Ethinyl Oestradiol (3×10^{-4} M)

Internal Standard (IS) Doxepin HCℓ

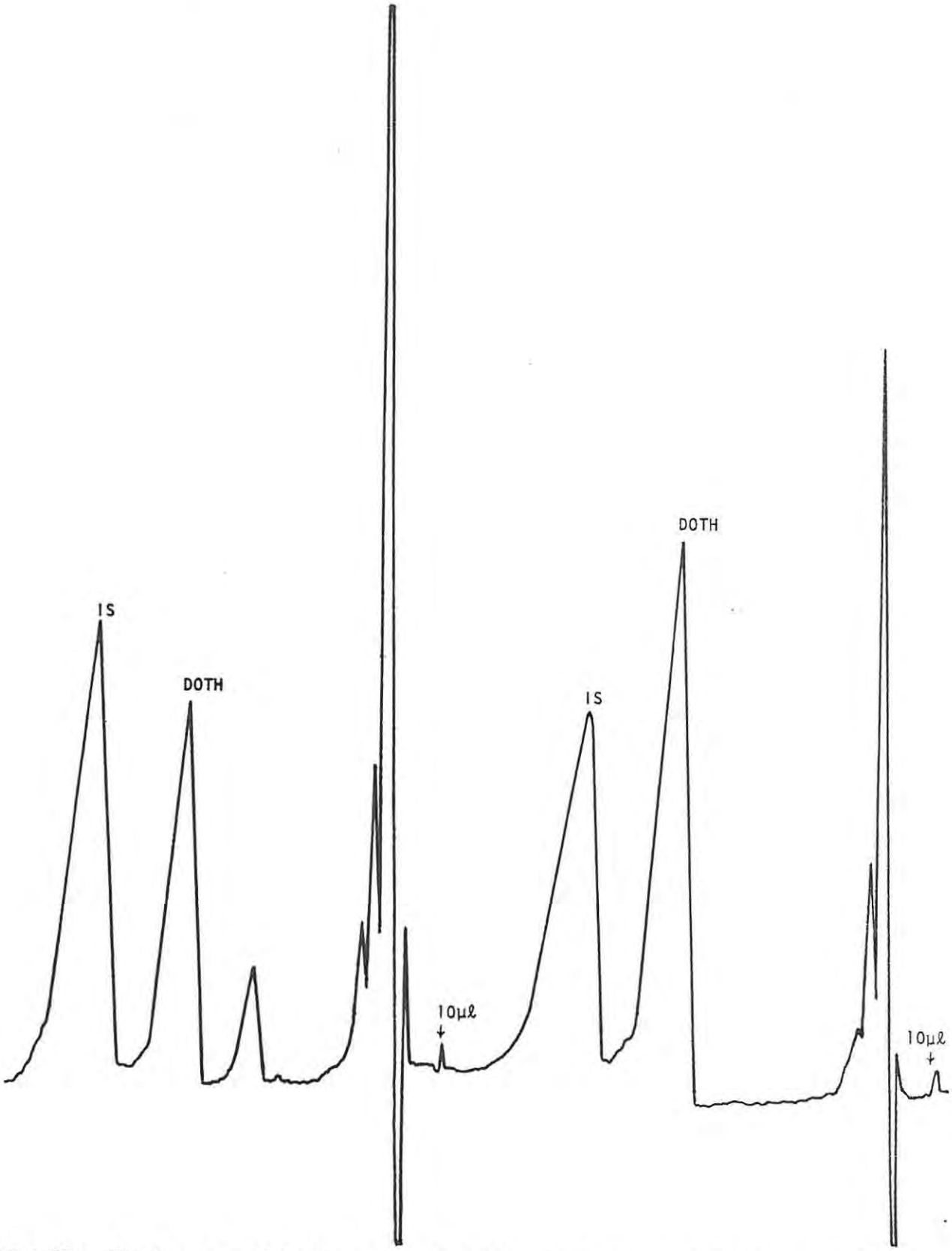


Figure 25a HPLC Spectrum of Dothiepin HCℓ (DOTH) Metabolised

Internal Standard (I.S.) Amitriptyline HCℓ

Figure 25b HPCL Spectrum of Dothiepin HCℓ (DOTH) Metabolised in the presence of Ethinyl Oestradiol (1×10^{-3} M)

Internal Standard (I.S.) Amitriptyline HCℓ

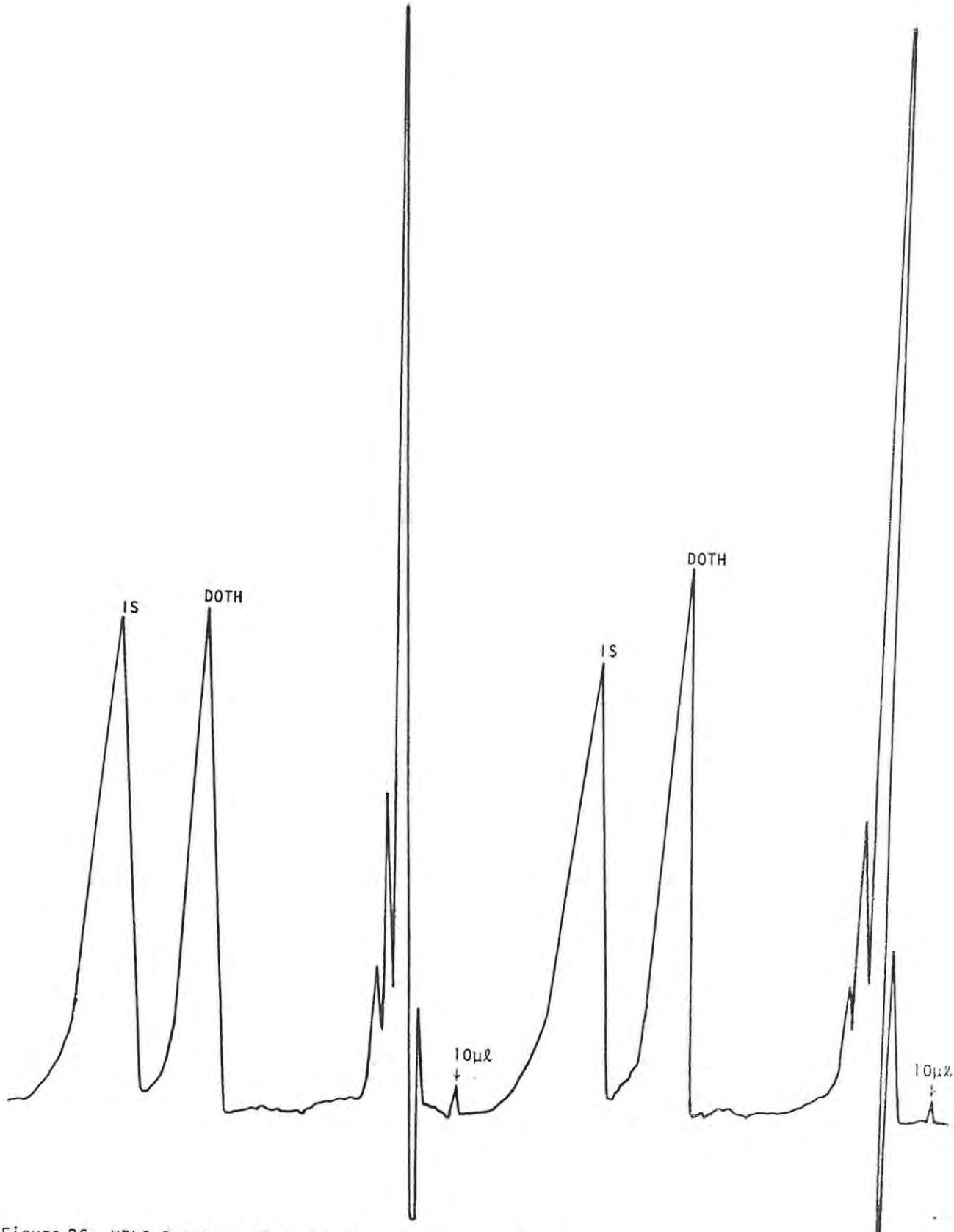


Figure 25c HPLC Spectrum of Dothiepin HCℓ (DOTH) Metabolised in the Presence of Ethinyl Oestradiol (1×10^{-4} M)

Internal Standard (I.S.) Amitriptyline HCℓ

Figure 25d HPLC Spectrum of Dothiepin HCℓ (DOTH) Metabolised in the Presence of Ethinyl Oestradiol (3×10^{-4})

Internal Standard (I.S.) Amitriptyline HCℓ

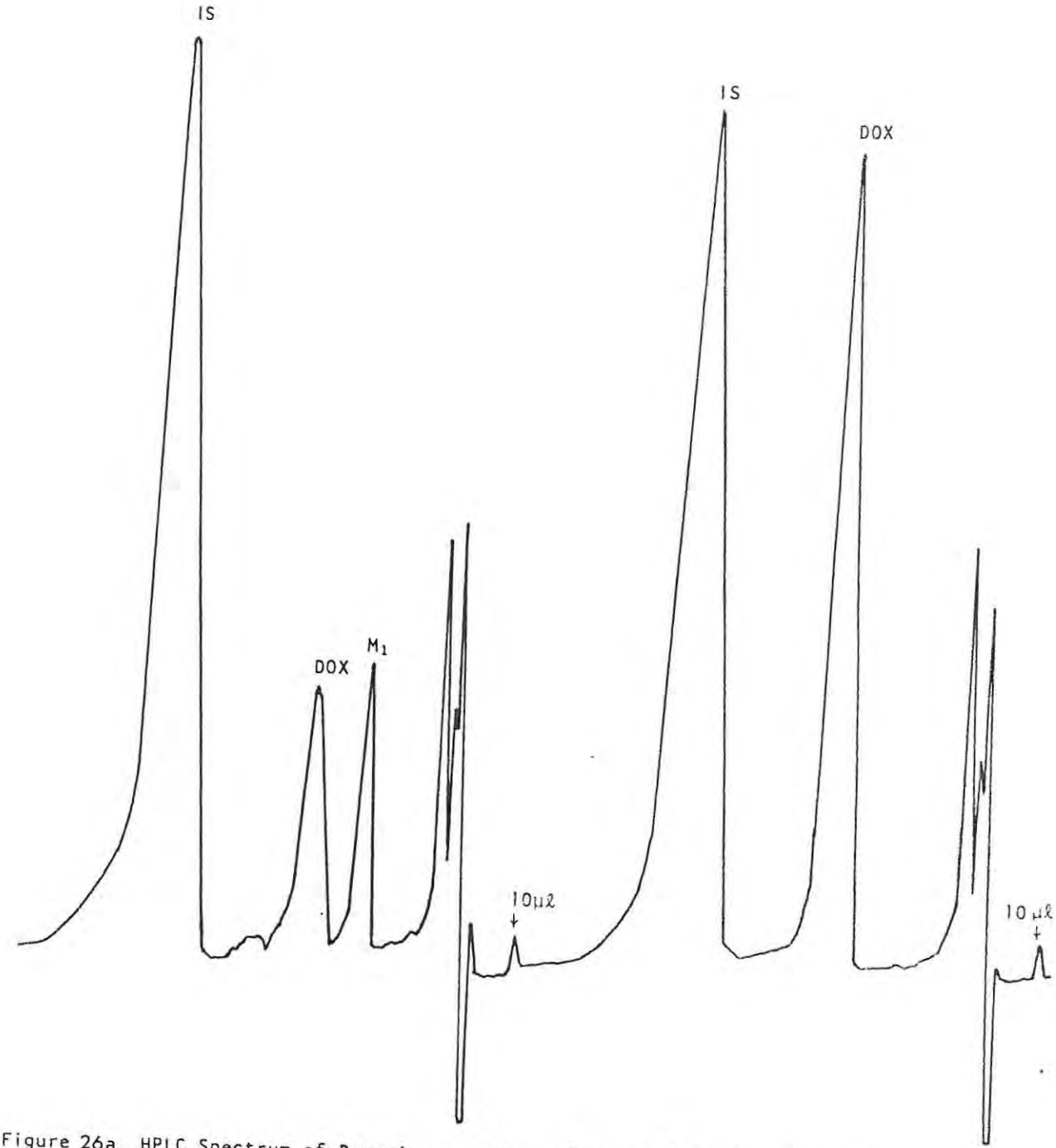


Figure 26a HPLC Spectrum of Doxepin HCℓ (DOX) Metabolised

Internal Standards (I.S) Amitriptyline HCℓ

Figure 26b HPLC Spectrum of Doxepin HCℓ (DOX) Metabolised in the presence of Ethinyl Oestradiol (1×10^{-3} M)

Internal Standards (I.S) Amitriptyline HCℓ

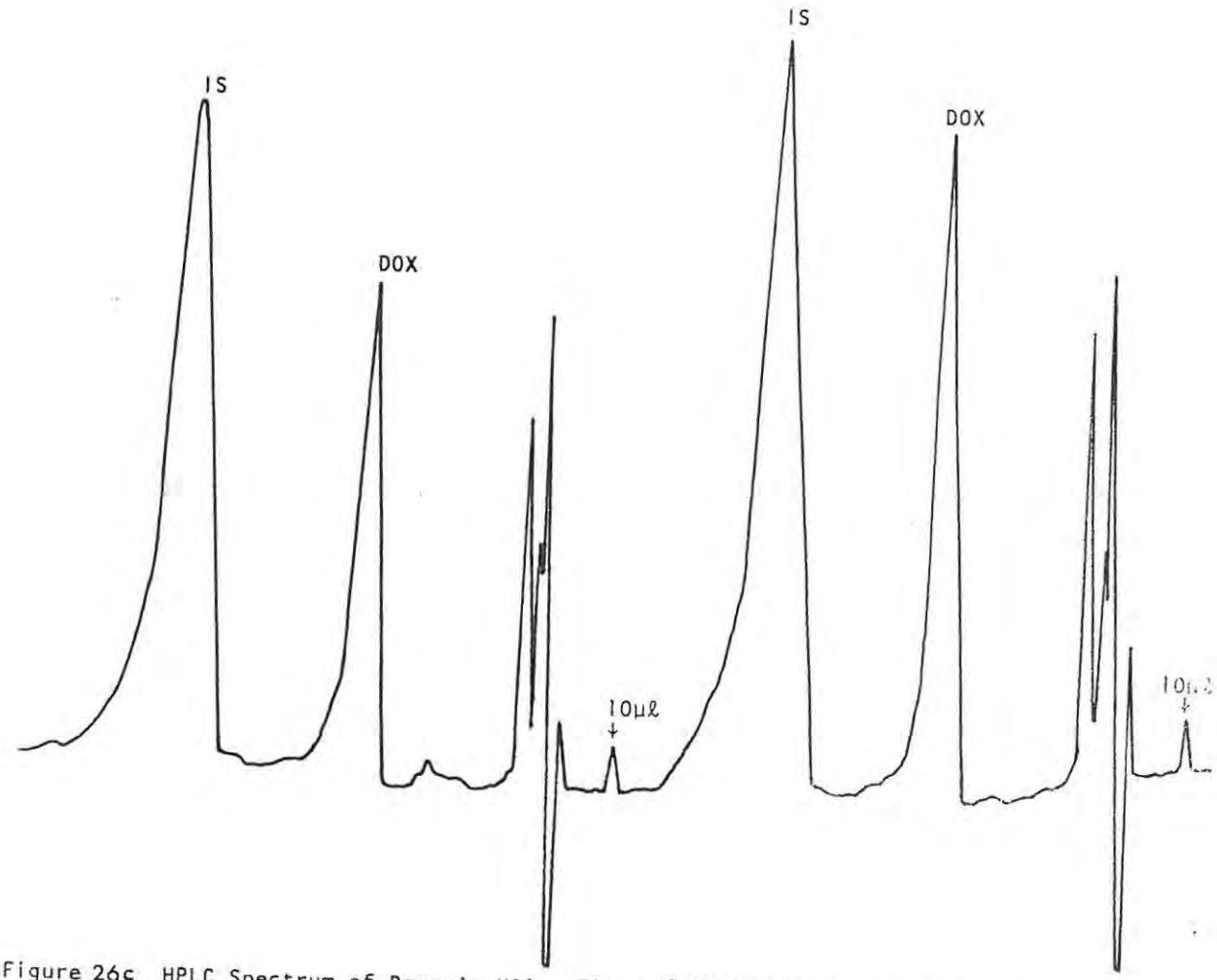


Figure 26c HPLC Spectrum of Doxepin HCℓ (DOX) Metabolised in the Presence of Ethinyl Oestradiol (1×10^{-4} M)

Internal Standard (I.S.) Amitriptyline HCℓ

Figure 26d HPLC Spectrum of Doxepin HCℓ (DOX) Metabolised in the Presence of Ethinyl Oestradiol (3×10^{-4} M)

Internal Standard (I.S.) Amitripyline HCℓ

TABLE 6

Concentration of Drugs used in Metabolic Reaction		Ratio of Peak Heights			% Metabolic Activity		
		A	B	C	A	B	C
A							
Imipramine HCℓ + Ethinyl Oestradiol							
a	1×10^{-4} M	-	0,73	1,72	1,79	100	100	100
b	1×10^{-4} M	1×10^{-3} M	1,46	2,20	2,20	0	0	0
c	1×10^{-4} M	3×10^{-4} M	1,26	2,07	2,11	28	22	20
d	1×10^{-4} M	1×10^{-4} M	1,21	2,03	2,03	35	35	41
B								
Amitriptyline HCℓ + Ethinyl Oestradiol							
a	1×10^{-4} M	-	0,50	0,40	0,38	100	100	100
b	1×10^{-4} M	1×10^{-3} M	1,13	0,95	1,04	0	0	0
c	1×10^{-4} M	3×10^{-4} M	0,89	0,80	0,80	38	27	36
d	1×10^{-4} M	1×10^{-4} M	0,75	0,68	0,67	60	50	56
C								
Dothiepin HCℓ + Ethinyl Oestradiol							
a	1×10^{-4} M	-	0,80	0,82	0,79	100	100	100
b	1×10^{-4} M	1×10^{-3} M	1,35	1,42	1,50	0	0	0
c	1×10^{-4} M	3×10^{-4} M	1,21	1,22	1,24	25	33	37
d	1×10^{-4} M	1×10^{-4} M	1,00	1,07	0,99	64	58	71
D								
Doxepin HCℓ + Ethinyl Oestradiol							
a	1×10^{-4} M	-	0,59	0,28	0,34	100	100	100
b	1×10^{-4} M	1×10^{-3} M	1,04	0,98	0,93	0	0	0
c	1×10^{-4} M	3×10^{-4} M	0,93	0,91	0,80	24	10	22
d	1×10^{-4} M	1×10^{-4} M	0,90	0,77	0,66	31	30	46
E								
Chlorimipramine HCℓ + Ethinyl Oestradiol							
a	1×10^{-4} M	-	0,30	0,27	0,26	100	100	100
b	1×10^{-4} M	1×10^{-3} M	0,85	0,85	0,87	0	0	0
c	1×10^{-4} M	3×10^{-4} M	0,78	0,75	0,77	13	17	16
d	1×10^{-4} M	1×10^{-4} M	0,59	0,65	0,64	48	34	38
F								
Trimipramine Maleate + Ethinyl Oestradiol							
a	1×10^{-4} M	-	1,13	1,00	0,99	100	100	100
b	1×10^{-4} M	1×10^{-3} M	1,94	1,87	1,93	0	0	0
c	1×10^{-4} M	3×10^{-4} M	1,51	1,56	1,60	53	36	35
d	1×10^{-4} M	1×10^{-4} M	1,42	1,29	1,24	64	66	73

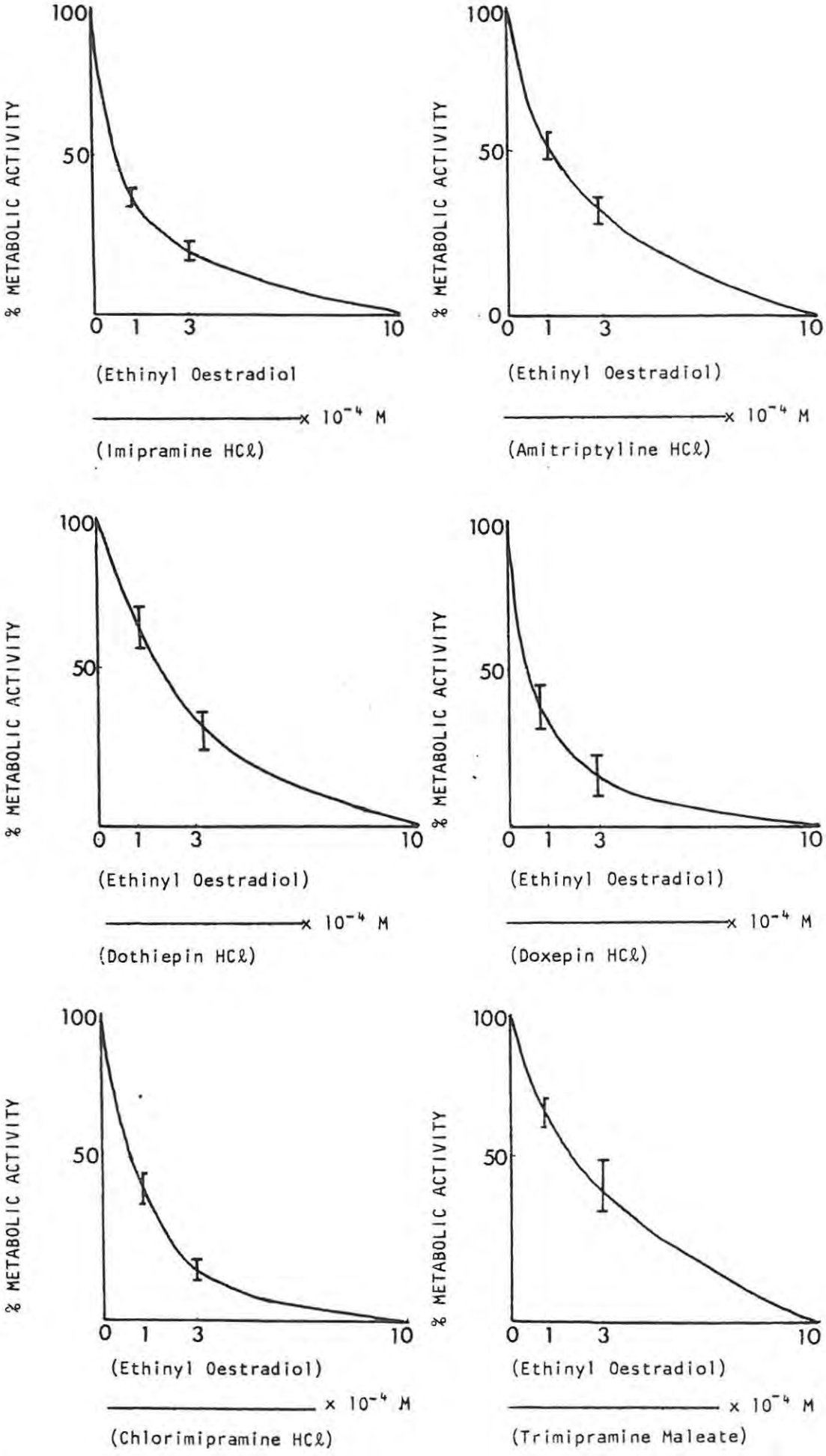

100
50
0
% METABOLIC ACTIVITY
0 1 3 10
(Ethinyl Oestradiol
× 10⁻⁴ M
(Imipramine HCℓ)
(Ethinyl Oestradiol)
(Amitriptyline HCℓ)
(Dothiepin HCℓ)
(Doxepin HCℓ)
(Chlorimipramine HCℓ)
(Trimipramine Maleate)

Fig. 27

6.4. A Study of the Anticholinergic Activity of Nomifensine, Tricyclic Antidepressives and their Derivatives

It has been noted that side effects experienced by people taking antidepressives are characteristic of anticholinergic activity. The aim of this study is to determine if these compounds possess anticholinergic activity, the order of affinity for and possible structure activity relationship with respect to anticholinergic effect.

Methods and Material

Metrohn flat bed recorder

Isotonic electronic transducer

Organ bath

Circulating water pump to maintain temperature.

Reagents

Tyrode solution

NaCℓ	8 g
KCℓ	0,2 g
$MgCℓ_2$	0,2 g
$CaCℓ_2$	0,2 g
NaH_2PO_4	0,05 g
$NaHCO_3$	1,0 g
Glucose	1,0 g
Distilled water to	1 litre

The Tyrode solution was freshly prepared each day. Two organ baths were used, both attached to a flat bed recorder, via an isotonic electronic transducer. A rat jujenum was prepared and then suspended in Tyrodes at 37°C and aerated.

A cumulative dose response curve was drawn using carbachol as

antagonist. Once two similar curves were obtained, the organ was washed, and the antagonist was added for 7 minutes so as to reach equilibrium with the tissue. Thereafter, another dose response curve was drawn and the shift of the curve was measured, and by means of tables the log affinity constants for the antagonists were determined.

Several determinations were made.

Results

All the drugs tested showed anticholinergic activity. The table shows the decreasing order of affinity for the cholinergic receptors in the rat jujenum.

	n	pA_2
Amitriptyline	6	8,0 ± 0,1
Protriptyline	6	8,0 ± 0,1
Trimipramine	6	7,9 ± 0,2
Dothiepin	6	7,8 ± 0,2
Nortriptyline	6	7,7 ± 0,2
Chlorimipramine	6	7,6 ± 0,2
Imipramine	6	7,6 ± 0,2
Desipramine	6	6,8 ± 0,2
Nomifensine	6	5,3 ± 0,3

The dibenzocycloheptane derivatives were found to be more potent than the dibenzazepine derivatives. In the dibenzocycloheptane series substitution of C-11 with S (Dothiepin) reduced potency. On the other hand methylation of the terminal nitrogen increased activity. The parent compounds possess a greater anticholinergic affinity as compared to their metabolites.

Conclusion

Maximum antagonistic activity depends on three factors.

1. Tricyclic skeleton
2. Side chain
3. The substitution on the terminal amine

1. The skeleton. Dibenzocycloheptane appears to confer optimal conformation and an alteration such as Dothiepin leads to a decrease in affinity.

2. Side chain. In the propyl side chain the double bond does not appear to be of particular significance comparing amitriptyline with protriptyline.

A substituent on the β C atom does, however, influence the affinity slightly, comparing trimipramine with imipramine.

3. Terminal amine. On the terminal amine of the side chain, especially when combined with dibenzazepine skeleton, the methyl substitution plays an important role.

DISCUSSION

The first experiments used rat hepatocytes as the metabolic agents and Premarin ^{R} as the inhibitor of metabolism. The results showed that Premarin ^{R} did not influence the metabolism of imipramine when using hepatocytes. However, what did come to light was how difficult it was to obtain hepatocytes. A long and extremely difficult surgical technique was required, and a watchful eye was needed to keep the rat sedated. The actual metabolic reactions were simple, as was the extraction method, although care was needed not to mix the extraction phases too much as an emulsion would form and this resulted in a reduced extraction of the parent compound and its lipophilic metabolites. A High Pressure Liquid Chromatograph was the detection apparatus. Using this apparatus it was quick and easy to detect the parent molecule and its metabolites. However, the instrument was very sensitive, hence the mobile phase had to be made carefully under recommended conditions, as a slight variation, for example, in pH, caused a marked difference in the shape and separation of the peaks. Although the actual runs for results were quick, a lengthy preparation was needed as well as thorough cleaning afterwards to ensure maximum column life.

A closer investigation was needed to explain why Premarin ^{R} did not inhibit the metabolism of imipramine. It could be because Premarin ^{R} possesses some conjugated oestrogens, therefore it is in a form which has a lower affinity than imipramine for the metabolic sites. Thus the body can get rid of Premarin ^{R} much more easily than imipramine, as it is already in a more water soluble form.

It therefore became necessary to use an unconjugated oestrogen and ethinyl oestradiol was chosen. Before another metabolic reaction was

performed, spectral studies of imipramine, ethinyl oestradiol and Premarin [R] were carried out. This was to establish if they all show the same kind of spectra and if so, this would indicate that they occupy the same metabolic binding sites. Rat microsomes were used as the spectrophotometer available did not lend itself to hepatocytes. The results showed that they all had a type I spectrum. Next was the determination of the saturation point of the microsomes using ethinyl oestradiol and Premarin [R] and then to see what influence these would have on the spectrum of imipramine. It was evident that more imipramine was required in both cases to obtain the same spectrum. More imipramine was used when comparing ethinyl oestradiol to Premarin [R]. A graphic plot was done and this showed that ethinyl oestradiol greatly affected the affinity of imipramine for the microsomes when compared to Premarin [R]. This, therefore, suggests that ethinyl oestradiol has a greater inhibitory effect than Premarin [R].

Further metabolic reactions were carried out using microsomes as the metabolic agents, as these were easier and less expensive to prepare. The same detection technique was used as for hepatocytes. Varying concentrations of ethinyl oestradiol were used to inhibit metabolism. Results distinctly showed that inhibition had taken place with a number of the tricyclic antidepressives used.

In addition a study of the anticholinergic activity of Nomifensine, tricyclic antidepressives and their derivatives was performed on a rat jujenum. This technique was slow and simple, although great care was needed when titrating the volumes of agonists and antagonists into the organ bath. Here results showed that the parent compounds had a greater affinity for the cholinergic receptors than their metabolites.

These results support the theory that the increased anticholinergic side effects experienced, are due to an inhibition of metabolism of tricyclic antidepressives, hence an increased level of parent compound in the blood stream, which has in turn a greater anticholinergic affinity.

A possible solution to the problem is to prescribe a newer antidepressive which has no or very little anticholinergic effect, or to choose a tricyclic antidepressive metabolite which has a lower pA_2 value compared to the parent molecule, such as desipramine.

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