IN-VITRO AND *IN-VIVO* RELATIONSHIPS IN DRUG METABOLISM

by

Diane E. MATTHEW, B.Sc. (Hons.) Manchester, M.P.S.

A thesis submitted to the

Victoria University of Manchester

for the degree of

Doctor of Philosophy

in the Faculty of Science

October 1987

DECLARATION

No portion of the work reported in this thesis has been submitted in support of an application for any other degree or qualification of this or any other University or institution of learning.

TO MY FAMILY

ACKNOWLEDGEMENTS

During the period of time spent on the production of this thesis, a large number of people have shown generosity with their time and experience. In recognition of this, I wish to thank the following people:

Dr. J.B. Houston, for his patience, encouragement and advice during the supervision of this research,

Dr. L. Aarons, for his contribution to the computation and data analysis,

Mrs. S.D. Warburton, for her technical assistance,

Mrs. V. Battleship, for her animal husbandry,

Mrs. E. Pearson and colleagues for keeping the laboratory spick and span,

My family for their help, support and encouragement,

Peter for his patience and long-sufferance,

and Professors A. Tallentire and M. Rowland for provision of research facilities within the Department of Pharmacy.

Finally I wish to thank the SERC for provision of a Studentship without which this work would not have been possible.

ABSTRACT

Relationships between *in-vitro* and *in-vivo* methods of assessing drug metabolizing capacity (*via* cytochrome P-450) have been examined in the rat. Both induction and inhibition studies were conducted and the merits and pitfalls of the *in-vitro* and *in-vivo* approaches were assessed.

In the induction studies, a wide range of drug metabolizing capacity was achieved in rats using the P450I inducer, β-naphthoflavone, and the P450IIB inducer, phenobarbitone. The specificity of the *in-vivo* clearance measurements, antipyrine and its metabolites, theophylline and tolbutamide, and the *in-vitro* measurements, cytochrome P-450 content, ECOD, MCOD, EROD and ALE activities, as markers for particular isoenzymes of cytochrome P-450 have been assessed and interrelationships investigated. Strong correlations were observed between theophylline clearance, 4-hydroxyantipyrine formation clearance, ECOD and EROD activities when rats were induced with β-naphthoflavone. Following phenobarbitone induction no improvement on the correlations obtained for antipyrine clearance was observed by using antipyrine metabolite formation clearances. The strongest correlations were observed between tolbutamide clearance, MCOD and ECOD activities. Dose ranging studies with the P450IV inducer, DEHP, indicated it to be a strong inducer of lauric acid hydroxylase, but to be a much weaker inducer of the other *in-vitro* and *in-vivo* parameters measured. It was therefore decided not to take these latter induction studies further.

In the inhibition studies, the potency of ketoconazole has been assessed using both *in-vitro* and *in-vivo* methods. *In-vitro*, it was shown that ketoconazole was 100 times more potent than cimetidine as an inhibitor and differences between two methods of defining potency (IC_{50} and K_i determinations) were investigated. *In-vivo* single dose inhibition

studies using the antipyrine breath test also indicated ketoconazole to be a potent inhibitor of antipyrine metabolism. Analysis of these data produced an estimate for K_i of 4.6 mg/Kg, however a decline in inhibitory effect was clearly evident during these experiments, indicating that ketoconazole was being rapidly eliminated from the system. Investigation of the pharmacokinetics of ketoconazole revealed it to exhibit dose dependent kinetics, and the plasma half-life was short. Ketoconazole pharmacokinetic parameters obtained in this study were used to produce a wide range of steady state concentrations and hence inhibition of antipyrine metabolism. Investigation of formation clearances of antipyrine metabolites demonstrated that ketoconazole was non-selectively inhibiting these pathways. K_i estimates for the three different pathways were in the range 0.8 - 1.2 mg/L. However in each case a significant portion of antipyrine metabolite capacity remained unaffected indicating that not all cytochrome P-450 activity was susceptible to inhibition by ketoconazole. There was good agreement between *in-vitro* and *in-vivo* K_i estimates, despite the use of different substrates in the two systems.

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CHAPTER 1

INTRODUCTION

Hepatic cytochrome P-450 mediated metabolism plays an important role in the modulation of drug action. Expression of the cytochrome P-450 system is under genetic control and is also affected by age, gender, diet, environmental chemicals, drugs and disease (Vesell, 1977, 1979). Hepatic drug metabolizing capacity reflects a balance of all these factors (Vesell, 1979), hence considerable attention has been focussed on these determinants and on realizing methods for prediction of individual drug metabolizing ability.

A major problem associated with research in this field is the existence of multiple forms of cytochrome P-450.

1.1 THE MULTIPLICITY OF THE CYTOCHROME P-450 SYSTEM

Cytochromes P-450 are terminal oxidases in an electron transport chain involved in the metabolism of both endogenous and exogenous compounds (Sato and Kato, 1984) and form a superfamily of NADPH-dependent monooxygenases (Nebert and Gonzalez, 1985). The existence of multiple forms of cytochrome P-450 has been established by various experimental techniques including SDS-polyacrylamide gel electrophoresis, amino acid sequence analysis, and catalytic assessments (Lu and West, 1980). Studies subsequent to these, involving cDNA probes (Leighton *et al.*, 1984), monoclonal antibodies (Roubi *et al.*, 1984a; 1984b) and kinetic analysis together with specific modulators of enzyme activity (Dieter and Johnson, 1982), have revealed subtle differences in structure and function between the various classes of cytochromes P-450. Although early grouping of cytochromes P-450 was by response to inducers, families defined by sequence homology are now recognised and may contain constitutive as well as inducible members (Leighton and Kemper, 1984).

Research in the area of cytochrome P-450 multiplicity has escalated but as yet no single system of nomenclature has been adopted. Indeed the science has become complicated by each research group introducing its own alternative nomenclature. Nebert *et al.* (1987) have proposed a system of nomenclature for the cytochrome P-450 gene superfamily based on a phylogenic classification. Members of families and subfamilies are defined as follows. Proteins in one gene family have less than or equal to 36% similarity with proteins in any of the other seven families and any proteins within the same subfamily have 70% or more similarity with each other. This system of nomenclature recommends Roman numerals for distinct gene families, capital letters for subfamilies, and Arabic numerals for individual genes (table 1.1). At present there is evidence for at least eight mammalian cytochrome P-450 gene families (Nebert *et al.*, 1987).

Table 1.1 summarises four families from the proposed cytochrome P-450 gene superfamily and shows that phenobarbitone-type inducers, together with ethanol, are different from other classes of inducers. Whereas the latter affect isoenzymes in families distinct from the major forms of cytochrome P-450, phenobarbitone-type inducers and ethanol affect one of the subfamilies of the major P450II family. Polycyclic aromatic hydrocarbon inducible cytochromes P-450 (P450I) are unusual in that there is a great deal of gene sequence homology between different mammalian species.

Knowledge of hepatic cytochrome P-450 isoenzyme composition and relative isoenzyme concentrations of an individual may provide valuable information about the potential responses of that individual to therapeutic drugs, of potential susceptibility to chemical carcinogens and of exposure to environmental pollutants if these induce cytochrome P-450. Beaune *et al.* (1986) have attempted to correlate a large number of monooxygenase activities with total cytochrome P-450 and

Table 1.1 The Cytochrome P-450 Gene Superfamily

Summary of the proposed nomenclature presented by Nebert *et al.* (1987) with relevance to the data presented in this thesis.

Family, subfamily and gene designation	Some of the existing names in the literature
P450I (Polycyclic aromatic hydrocarbon-inducible)	
Only one subfamily	
P450IA1	Rat c, mouse P_1, rabbit form 6, human P_1
P450IA2	Rat d, mouse P_3, rabbit form 4, human P_3
P450II (Major)	
Five subfamilies	
1)P450IIA:	
P450IIA1	Rat a
P450IIA2	Human P450(1)
2)P450IIB: (Phenobarbitone-inducible)	
P450IIB1	Rat b, rabbit form 2
P450IIB2	Rat e
3)P450IIC:	
P450IIC1	Rabbit PBc1
P450IIC2	Rabbit PBc2, k
P450IIC3	Rabbit PBc3, 3b
P450IIC4	Rabbit PBc4, 1-8
P450IIC5	Rabbit form 1
P450IIC6	Rat PB1
P450IIC7	Rat f
P450IIC8	Human form 1
P450IIC9	Human mp
P450IIC10	Chicken PB15
4)P450IID:	
P450IID1	Rat (and human) db1
P450IID2	Rat db2
5)P450IIE: (Ethanol-inducible)	
P450IIE1	Rat j, rabbit form 3a, human j
P450III (Steroid-inducible)	
Only one subfamily	
P450IIIA1	Rat pcn1
P450IIIA2	Rat pcn2
P450IIIA3	Human HLp, nf
P450IV (Peroxisomal proliferator-inducible)	
Only one subfamily	
P450IVA1	Rat LAω

concentrations of isolated isoenzymes, and tentatively grouped these into five major categories. Further studies are needed, however, since correlations produced between monooxygenase activity and particular isoenzymes were not generally predictive ($r < 0.77$, $r^2 < 0.59$). Knowledge of the contribution that each isoenzyme makes to the metabolism of particular drugs would also facilitate prediction. At present, such studies are confounded by the difficulty in obtaining suitable human liver samples, lack of knowledge with respect to the exposure of the donor to factors influencing drug metabolism and limited knowledge of human cytochrome P-450 isoenzyme composition. Thus, there is wide scope for animal studies where environmental and genetic factors are well defined.

1.2 ASSESSMENT OF DRUG METABOLIZING ABILITY

Patient variability in drug response can be attributed in part to differences in drug metabolizing capacity which in turn depend on the total amount of drug metabolizing enzymes (Davies *et al.*, 1973) and also on the proportion of each cytochrome P-450 form (Comai and Gaylor, 1973). Animal studies have shown that the various isoenzymes of cytochrome P-450 exhibit differences in substrate specificity (Lu *et al.*, 1973), in regio-selectivity of metabolism (Wiebel *et al.*, 1975), in their response to inducers and/or inhibitors (Haugen *et al.*, 1976, Ullrich *et al.*, 1975) and with age (Atlas *et al.*, 1977), gender (von Bahr *et al.*, 1977), tissue (Atlas *et al.*, 1977), and species (Thomas *et al.*, 1976).

Differences in the rates of drug metabolism are reflected by large differences in plasma clearance and hence steady state plasma concentrations during long term therapy. Since many drugs have narrow therapeutic plasma concentration ranges, such variability will give rise either to undertreatment or overdosage in individual patients. The

identification of such problems led to the concept of therapeutic drug monitoring during long term treatment with such drugs as anticonvulsants, antiarrhythmics, antibiotics, and theophylline. Since drug dosage will need to be altered following exposure to or removal of any influencing factor of drug metabolism, then the sources of the variation should ideally be identified in order to be alert to potential interactions. Methods for investigating such variability have been the subject of a number of reviews (Breimer, 1983; Vessell and Penno, 1983). Various *in-vivo* probe compounds have also been used in the assessment of drug metabolizing capacity (Park, 1982) and are the subject of this section.

1.2.1 *In-vivo* Methods

Compounds used as *in-vivo* probes of drug metabolizing capacity can be considered either as exogenous drug substances or endogenous compounds.

1.2.1.1 *Endogenous Markers of Drug Metabolizing Activity*

Monitoring of endogenous compounds has a number of advantages over determination of xenobiotic metabolism. Firstly, with such compounds there is no perturbation of drug metabolizing enzymes which may occur on administration of an exogenous compound. Secondly, since a number of these substances are monitored in urine rather than plasma, then the method can be non-invasive. Three compounds, γ-glutamyl transpeptidase, D-glucaric acid and 6 β-hydroxycortisol, have been studied for their suitability in the assessment of drug metabolism.

The discovery that plasma levels in man of γ-glutamyl transpeptidase (Hildebrandt *et al.*, 1975; Ohnhaus *et al.*, 1983; Rosalki *et al.*, 1971, Satoh *et al.*, 1982; Walter *et al.*, 1982; Whitefield *et al.*, 1972) and the urinary excretion of D-glucaric acid (Davis *et al.*, 1974; Hildebrandt *et al.*, 1975; Hunter *et al.*, 1971; Hunter and Chasseaud,

1976; Marsh, 1963a, 1963b) and 6 β-hydroxycortisol (Kuntzman *et al.*, 1968; Poland *et al.*, 1970) were elevated by inducing agents, promoted investigation of the use of such compounds as probes of drug metabolizing capacity. Correlations have been found between plasma γ-glutamyl transpeptidase and the elimination rates of antipyrine, phenylbutazone and warfarin (Smith and Rawlins, 1974); urinary excretion of D-glucaric acid and antipyrine half-life (Hunter *et al.*, 1974) and urinary excretion of 6 β-hydroxycortisol and antipyrine clearance (Ohnhaus and Park, 1979; Ohnhaus *et al.*, 1983).

The main problem associated with the use of such indices of drug metabolizing ability is that pathological conditions, which have no influence on drug metabolism, can influence the plasma levels of γ-glutamyl transpeptidase (Whitefield *et al.*, 1972), and the urinary excretion of D-glucaric acid (Park, 1982) and 6 β-hydroxycortisol (Park, 1981).

1.2.1.2 ***Exogenous Compounds as Markers of Drug Metabolizing Capacity***

Many drugs, including antipyrine, aminopyrine, amylobarbitone, diazepam, hexobarbitone, phenacetin, phenylbutazone, quinine, theophylline, tolbutamide and warfarin (Park, 1981), have been used as model compounds for detecting changes in drug metabolizing capacity. The disposition of drug substances greatly influences their subsequent rate of metabolism. In this respect drug substances can be classified by hepatic clearance as follows.

1. drugs with low clearance and minimal protein binding (e.g. antipyrine and theophylline)
2. drugs with low clearance and high protein binding (e.g. tolbutamide, warfarin and phenylbutazone)
3. high clearance drugs showing blood flow dependent hepatic extraction (e.g. lignocaine and propranolol).

Drugs from any of these groups may be used as model compounds provided they are metabolized mainly by the liver. Low clearance, minimal binding drugs are the simplest to use since their kinetics are independent of variables such as protein binding and hepatic blood flow. The metabolism of low clearance highly protein bound drugs is affected by the degree of binding, thus protein binding measurements must also be made when studying such kinetics. Highly cleared drugs show metabolism rates which are limited by hepatic blood flow (Rowland and Tozer, 1980), hence the degree of protein binding does not dramatically affect clearance.

Antipyrine has been extensively used to identify many of the factors which determine drug metabolizing capacity. Attempts to predict the disposition of therapeutic drugs from the disposition of antipyrine, however, have been less successful. Many of these predictive studies have tried to produce correlations using half-life data, but clearance is thought to be a more appropriate parameter to use.

1.2.1.2.1 *Intrinsic Clearance*

Intrinsic clearance (Cl_{int}) provides a measure of the drug metabolizing capacity of an organ and is based upon the unbound concentration of a drug which can be almost totally eliminated by the liver. Cl_{int} can be calculated from *in-vivo* data. If a drug is completely metabolized, then drug clearance (CL_H) equates to (Cl_{int}). CL_H can generally be calculated as shown in equation 1.1 and hence intrinsic clearance may be estimated:

$$CL_H = Cl_{int} = \frac{Dose}{AUC_{iv}} \qquad \text{equation 1.1}$$

AUC_{iv} - area under the iv blood/plasma concentration-time curve

This, the simplest situation, holds for low clearance, minimal binding drugs and can be used for low clearance, high binding drugs by including a term for the unbound fraction (f_u) (equation 1.3), necessary since the measured intrinsic clearance is influenced by the unbound concentration.

$$CL_{int} = \frac{CL_H}{f_u} \qquad \text{equation 1.2}$$

In order to determine the intrinsic clearance of highly cleared drugs, hepatic blood flow must also be considered. Thus, using the well-stirred model of Pang and Rowland (1977);

$$CL_H = \frac{f_u . CL_{int} . Q_H}{Q_H + (f_u . CL_{int})} \qquad \text{equation 1.3}$$

Also $$CL_H = Q_H . E \qquad \text{equation 1.4}$$

CL_H - hepatic clearance

Q_H - hepatic blood flow

E - hepatic extraction ratio

Equations 1.3 and 1.4 can be combined;

$$CL_{int} = \frac{CL_H}{f_u . (1 - E)} \qquad \text{equation 1.5}$$

If a drug is 100% cleared by metabolism in the liver, and is orally administered;

$$CL_H = \frac{F . Dose_{(oral)}}{AUC_{(oral)}} \qquad \text{equation 1.6}$$

also $F = 1 - E$ equation 1.7

By combining equations 1.6 and 1.7;

$$CL_{int} = \frac{Dose_{(po)}}{f_m . AUC_{(po)}}$$ equation 1.8

1.2.1.2.2 *Metabolite Formation Clearance*

Measurement of the clearance of model compounds provides information about the overall drug metabolizing capacity of individuals. It is known, however, that the cytochrome P-450 system of the liver is composed of several different isoenzymes each having different inter- and intra-substrate specificities, each with their own regulatory control and differently affected by inducing agents (Lu and West, 1980; Nebert, 1979). Metabolite formation clearances, rather than the overall clearance of drugs may provide a means of separately assessing the activities of these different forms of cytochrome P-450 (Breimer, 1983; Danhof, 1980).

Formation clearances of the metabolites of a drug are determined using the fraction of each individual metabolite formed (f_m) relative to dose of drug administered, together with the total clearance of the parent drug (CL):

$$CL_m = CL . f_m$$ equation 1.9

1.2.1.2.3 *Antipyrine*

Once used as an antipyretic and analgesic agent, antipyrine is nowadays used mainly as a research tool (Danhof and Tauniseen, 1984). In the late 1960's Welch *et al.* (1967) used studies in dogs and monkeys to introduce the concept of using the elimination rate of antipyrine from plasma as

an indicator of the activity of the enzymes by which it is metabolized. Vessell and co-workers (Vessell and Page, 1968) subsequently used antipyrine for human studies and since then antipyrine elimination has been extensively used to investigate many factors influencing drug metabolism (Vessell, 1979).

1.2.1.2.3.1 *Metabolism*

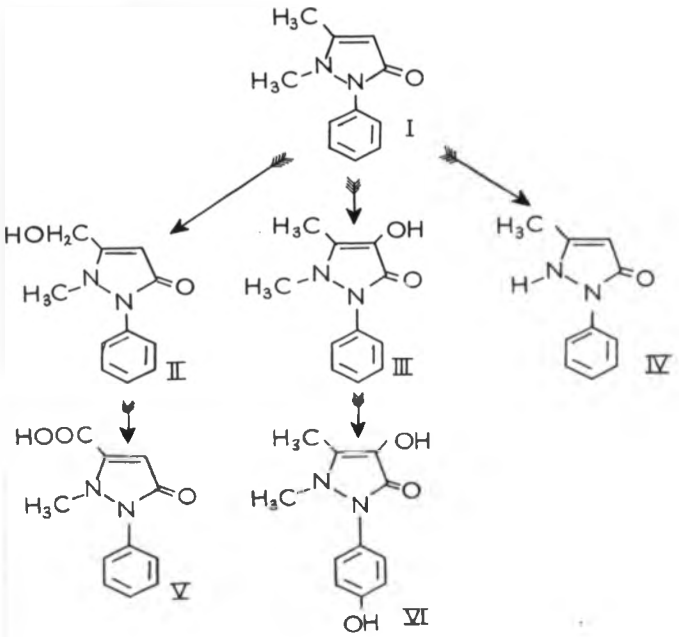
Antipyrine is metabolized by at least three independent metabolic pathways (figure 1.1) resulting in the formation of 4-hydroxyantipyrine (4H), norantipyrine (N) and 3-hydroxymethylantipyrine (3H) (Danhof *et al.*, 1982a). Minor metabolites have been identified as 3-carboxy-antipyrine, a product of the oxidation of 3-hydroxymethylantipyrine, and 4,4'-dihydroxyantipyrine a product of aromatic ring hydroxylation of 4-hydroxyantipyrine (Bottcher *et al.*, 1982; Danhof *et al.*, 1982a; Inaba *et al.*, 1981). Qualitatively, these metabolites are produced both in rats and man. There are, however, quantitative differences between these species.

1.2.1.2.3.2 *Pharmacokinetics*

Antipyrine is noted for its rapid and complete absorption from the gastro-intestinal tract, its distribution into total body water with negligible binding to tissue or plasma proteins and its almost complete metabolism in the liver with low extraction ratio and negligible renal elimination (Vessell, 1979). These characteristics indicate it to be a low clearance, minimal binding drug and therefore its metabolism is independent of liver blood flow.

Figure 1.1 Metabolic Pathways of Antipyrine

I - antipyrine
II - 3-hydroxymethylantipyrine
III - 4-hydroxyantipyrine
IV - norantipyrine
V - 3-carboxyantipyrine
VI - 4,4'-dihydroxyantipyrine


I
II
III
IV
V
VI

1.2.1.2.3.3 *Use as a Model Compound*

Antipyrine is widely used to reflect hepatic drug metabolism in man (Andreasen *et al.*, 1974; Vessell, 1979). As a low clearance, low binding drug its elimination is determined largely by hepatic oxidizing enzyme activity and therefore may serve as a quantitative measure of liver function (Andreasen *et al.*, 1974; Homeida *et al.*, 1979). Antipyrine has been used extensively, therefore, to investigate the postulate that such metabolism of model compounds might predict the *in-vivo* disposition characteristics of therapeutic drugs. It has also been used to identify sources of variability in drug metabolizing ability. The advantages and limitations of this latter application have been extensively reviewed (Vessell, 1979; Vessell and Penno, 1983).

The use of antipyrine metabolism as a universal predictor of *in-vivo* disposition characteristics has rarely produced predictive correlations. Although the usefulness of half-life as a parameter has been questioned, the use of the preferred parameter, clearance, has not improved such correlations. As evidence for the existence of multiple forms of cytochrome P-450 increased it became clear that different isoenzymes had different substrate specificities (Wang *et al.*, 1983). Use of the pharmacokinetic data for one compound to predict the disposition of the wide range of therapeutic compounds which are metabolized is therefore unrealistic. Only when the same isoenzymes are involved in the metabolism of the two compounds can the response to one satisfactorily predict the metabolism of the other.

The postulate of Huffman *et al.* (1973), that different enzymes are involved in the formation of the various metabolites of antipyrine, has been confirmed by other groups (Boobis *et al.*, 1981; Danhof *et al.*, 1979; Inaba *et al.*, 1980; Kahn *et al.*, 1982; Rhodes and Houston, 1983b). Breimer (1983) suggested that the use of metabolite clearances rather than total clearances might improve relationships between the

metabolism of two compounds (Breimer, 1983). This has subsequently been confirmed (Tenhimsen et al 1986; Van der Graaff *et al.*, 1983b).

Some selectivity has been observed in the formation clearances/rates of the metabolites of antipyrine with various inducing and inhibiting agents (table 1.2). B-NF-mediated cytochrome P450I induction of the pathways of antipyrine, for example, was found to be selective for 4-hydroxylation and N-demethylation, whereas 3-hydroxylation was reduced. PB-mediated cytochrome P450IIB induction on the other hand, non-selectively increased the formation rates of all three metabolites (Rhodes and Houston, 1983b). Various inducers and inhibitors have been observed to differentially affect the metabolic pathways of antipyrine in man. Inhibition of antipyrine metabolism by propranolol was found to be most pronounced for 3-hydroxymethylantipyrine, although in some individuals also a decrease in formation clearance of 4-hydroxyantipyrine and norantipyrine was observed (Bax *et al.*, 1981). Following treatment with phenytoin and carbamazepine, 3-hydroxymethylantipyrine and 4-hydroxyantipyrine formation clearances were selectively increased, whilst norantipyrine formation clearance was unaffected (Shaw *et al.*, 1985).

1.2.1.2.4 *Theophylline*

Theophylline is used as a bronchodilator for the symptomatic and prophylactic treatment of asthma and for reversible bronchoconstriction. Its pharmacological action is to inhibit the enzyme phosphodiesterase and leads to an increase in the intracellular concentration of cyclic adenosine monophosphate (cAMP) to cause relaxation of bronchiolar smooth muscle and relief of bronchospasm. Since it has a narrow therapeutic index serum concentrations are determined when treatment is commenced in order to optimise therapy. Modest changes in theophylline clearance may result in insufficient bronchodilation or the appearance of toxic side-

Table 1.2 Selectivity of Drugs, Interacting with Antipyrine, towards the Formation Clearance, Formation Rate or Urinary Excretion of Antipyrine Metabolites

Interacting Agent	Effect	Species	Effect on AP Metabolites	References
Aroclor 1254	Induction	rat	↓ 3H, ↑ 4H, ∞ N	Shaw & Houston 1986
β-NF	"	"	↓ 3H, ↑ 4H, ↑ N	Rhodes & Houston 1983b
3-MC	"	"	↓ 3H, ↑ 4H, ↑ N	Teunissen *et al.* 1983a
Antipyrine	"	"	non-selective	Shaw *et al.* 1986
PB	"	"	non-selective	Teunissen *et al.* 1983b
PCN	"	"	non-selective	Shaw *et al.* 1986
Promethazine	"	"	non-selective	Shaw *et al.* 1986
*α-NF	Inhibition	rat	∞ 3H, ∞ 4H, ↓ N	Rhodes *et al.* 1984
*SKF 525A	"	"	↓ 3H, ↑ 4H, ∞ N	Rhodes *et al.* 1984
Cimetidine	"	"	non-selective	Adedoyin *et al.* 1987
*Metyrapone	"	"	non-selective	Rhodes *et al.* 1984
Carbamazepine	Induction	man	↑ 3H, ↑ 4H, ∞ N	Shaw *et al.* 1985
Phenytoin	"	"	↑ 3H, ↑ 4H, ∞ N	Shaw *et al.* 1985
Rifampicin	"	"	∞ 3H, ∞ 4H, ↑ N	Teunissen *et al.* 1984
Pentobarbitone	"	"	non-selective	Danhof *et al.* 1982b
Phenobarbitone	"	"	non-selective	Dossing *et al.* 1983
Sulphinpyrazone	"	"	non-selective	Staiger *et al.* 1983
Propranolol	Inhibition	man	↓ 3H, ∞ 4H, ∞ N	Bax *et al.* 1981
Cimetidine	"	"	non-selective	Teunissen *et al.* 1985c
Verapamil	"	"	non-selective	Bach *et al.* 1986
OCS	"	"	non-selective	Teunissen *et al.* 1982

α-NF - α-naphthoflavone
β-NF - β-naphthoflavone
3-MC - 3-methylcholanthrene
OCS - oral contraceptive steroids
PB - phenobarbitone
PCN - pregnenolone 16α-carbonitrile
* urinary excretion data

effects. A large number of genetic, pathophysiological and environmental factors have been identified, which influence theophylline pharmacokinetics (Gardner and Jusko, 1984; Ogilvie, 1978; Powell, 1984). Concurrent administration of drugs may also affect theophylline clearance (Jonkman and Upton, 1984).

Realisation of the multiplicity of the cytochrome P-450 system led to a search for marker compounds with specificity for particular isoenzymes of cytochrome P-450 (Kellermann and Luyten-Kellermann, 1978). Since the metabolism of theophylline had been found to be accelerated by cigarette smoking, it was suggested that this drug might be used as a marker of cytochromes P450I in man and rat (Breimer *et al.*, 1984; Jenne, 1982; Kappas *et al.*, 1976).

1.2.1.2.4.1 *Metabolism*

Theophylline (1,3-methylxanthine) is extensively metabolized in man and animals (figure 1.2) to 3-methylxanthine and 1-methyluric acid and 1,3-dimethyluric acid (Brodie *et al.*, 1952; Cornish and Christman, 1957; Jenne *et al.*, 1976; Weinfeld and Christman, 1953) by the hepatic mixed function oxidase system (Lohmann and Miech, 1976). These metabolites account for up to 90% of an administered dose, with only about 10% being recovered as unchanged drug.

1.2.1.2.4.2 *Pharmacokinetics*

Theophylline pharmacokinetics has been widely studied in the rat (Tenuissen *et al.*, 1985a; Williams *et al.*, 1979) dog (Clozel *et al.*, 1981; Tse and Szeto, 1982) cat (McKiernan *et al.*, 1983) guinea-pig (Madsen and Ribel, 1981), horse (Errecalde *et al.*, 1985; Ingvast-Larsson *et al.*, 1985), rabbit (Canada *et al.*, 1986) and man (Bonatti and Garattini, 1984; Chrzanowski *et al.*, 1977; Ellis *et al.*, 1976; Mitenko and Ogilvie, 1972; Ogilvie, 1978). Although plasma concentration-time

Figure 1.2 Metabolic Pathways of Theophylline

I - Theophylline

II - 3-methylxanthine

III- 1,3-dimethyluric acid

IV - 1-methylxanthine

V - 1-methyluric acid.

I
II
III
IV
V

profiles for theophylline following administration of therapeutic doses have been described by linear kinetics following administration of therapeutic doses (Tang-Liu *et al.*, 1982), various studies have suggested that theophylline exhibits capacity limited kinetics (Weinberger and Ginchansky, 1977). When plasma drug concentrations above 20mg/L are reached, then log-linear decay curve is no longer observed; rather the curve follows expected capacity limited kinetics (Jenne *et al.*, 1977; Kadlec *et al.*, 1978). Human clearance of theophylline is highly variable with values of between 8 and 200 ml/Kg/h being observed (Ogilvie, 1978).

Lohmann and Miech (1976) reported a half-life of theophylline in rats of about 6 h, whilst Tenhimsen *et al.* (1981) found doses of 20mg to produce a similar half-life, lower doses (5mg) have half-lives of only 70 min. In a more extensive study it was found that AUC increased disproportionately with theophylline dose, thus indicating capacity limited metabolism (Tenhimsen *et al.*, 1985a).

1.2.1.2.4.3 *Use as a Model Compound*

Polycyclic aromatic hydrocarbons (PAH) are widely dispersed in nature and may influence the disposition of those drugs which are metabolized by PAH inducible isoenzymes. Identification and use of specific isoenzyme probes may help to determine whether these isoenzymes are also involved in the metabolism of other drugs.

Interest in the use of theophylline as a probe of drug metabolism stems from knowledge that metabolism can be enhanced by 3-methylcholanthrene in the mouse (Batlach and Tozer, 1980) and rat (Tenhimsen *et al.*, 1986; Williams *et al.*, 1979). In man, diets containing charcoal-broiled beef (Kappas *et al.*, 1978) and tobacco and marijuana smoking (Jusko *et al.*, 1978; Jusko *et al.*, 1979; Powell *et al.*, 1977), both of which may involve the intake of polycyclic aromatic

hydrocarbons (PAHs) (cytochrome P450I inducers), shorten theophylline elimination half-life. Campbell *et al.* (1987) observed that theophylline and ethoxyresorufin competitively inhibited each others metabolism in human liver microsomes and that EROD activity correlated highly (r=0.91) with theophylline 1-demethylation. Since ethoxyresorufin is a substrate for cytochrome P450I, then these data provide further evidence for the involvement of cytochrome P450I in the metabolism of theophylline.

Other studies, however, have indicated that PB-inducible forms of cytochrome P-450 are also involved in the metabolism of theophylline (Jonkman and Upton, 1984). Interestingly, Acheson *et al.* (1987) observed that the effects of anticonvulsant drugs and polycyclic aromatic hydrocarbons on the metabolism of antipyrine or theophylline, were not additive.

1.2.1.2.5 *Tolbutamide*

Tolbutamide is an oral hypoglycaemic agent used for the chronic treatment of diabetes mellitus of the maturity-onset type. The hypoglycaemic effect of tolbutamide only occurs when the β-cells of the islets of Langerhans are intact and some functional capacity is retained. Since tolbutamide has no influence on glucose tolerance, it is thought to stimulate the pancreas to produce more insulin.

1.2.1.2.5.1 *Metabolism*

After oral administration to humans, about 85% of the dose is excreted in the urine as the inactive carboxy (1-butyl-3-p-carboxyphenylsulphonylurea) and hydroxymethyl metabolites (1-butyl-3-p-hydroxymethylphenylsulphonylurea) (Thomas and Ikeda, 1966). Tolbutamide is oxidized to hydroxytolbutamide and then to carboxytolbutamide. Its usefulness as a probe of drug metabolism is complicated by the fact that it undergoes extensive sequential metabolism (figure 1.3) and shows high

protein binding. Effects due to alteration in binding must therefore be differentiated from those due to changes in hepatic metabolic activity.

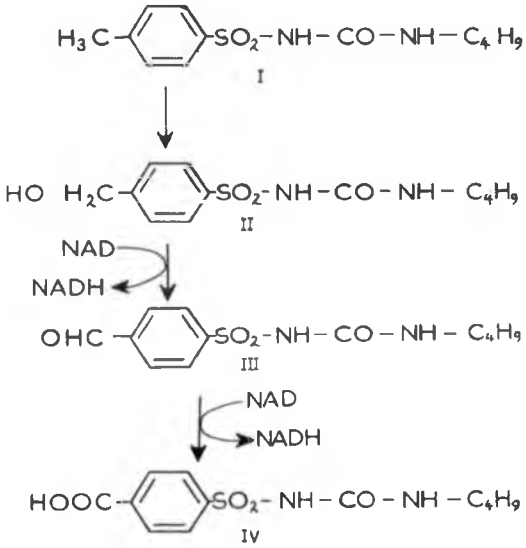
The metabolism of tolbutamide has been extensively studied and pronounced species differences noted. The dog is reported to excrete p-tolylsulphonamide and p-tolylsulphonylurea (Mohnike *et al.*, 1958; Wittenhagen *et al.*, 1959). Reports of metabolites of tolbutamide in the cat have been conflicting. Larsen and Madsen (1962) suggested that hydroxy-tolbutamide was the primary metabolite whereas Wittenhagen *et al.* (1959) indicated that metabolism was similar to that in the dog. In the rat, 80% of an orally administered dose of tolbutamide is excreted in urine, predominantly as hydroxytolbutamide. Small amounts of carboxytolbutamide and p-tolylsulphonylurea (about 5% of dose excreted) were also detected. In man, however, carboxy-tolbutamide (67%) is present in urine to a greater extent than hydroxy-tolbutamide (33%) (Thomas and Ikeda, 1966). Tagg *et al.* (1967), have shown that tolbutamide is converted in the liver to hydroxytolbutamide by NADPH-linked microsomal enzymes. The oxidation pathway appears to be almost quantitative in the rat and man (Rowland and Matin, 1973; Thomas and Ikeda, 1966). Hydroxytolbutamide is further metabolized to carboxy-tolbutamide presumably via tolbutamide aldehyde, by alcohol and aldehyde dehydrogenases of the cytosol (Hansen and Christensen, 1977; McDaniel *et al.*, 1969) (figure 1.3). It can therefore be seen that oxidation of tolbutamide to hydroxytolbutamide is rate limiting for the elimination of the drug and its metabolites (Rowland and Matin, 1973; Hansen and Christensen, 1977).

1.3.1.2.5.2 *Pharmacokinetics*

Following ip administration in rats (50mg/Kg) tolbutamide half-life was estimated as 129 min, clearance as 1.2 ml/min/Kg, apparent volume of distribution as 0.21 L/Kg and AUC as 513 h x mg/L (Back *et al.*, 1984).

Figure 1.3 Metabolic Pathway of Tolbutamide

I - tolbutamide
II - hydroxytolbutamide
III - tolbutamide aldehyde
IV - carboxytolbutamide


H3C
SO2-NH-CO-NH-C4H9
I
HO H2C
SO2-NH-CO-NH-C4H9
II
NAD
NADH
OHC
SO2-NH-CO-NH-C4H9
III
NAD
NADH
HOOC
SO2-NH-CO-NH-C4H9
IV

Concentration-time profiles demonstrated monoexponential kinetics in rats.

In a human study, Matin *et al.*, (1974) found the decline in plasma concentration following iv administration of tolbutamide, to be biphasic. The terminal half-life was 7.2 ± 0.8 h and the apparent volume of distribution, 7.3 ± 0.9 L. Andreasen and Vessell (1974) compared the oral and iv routes of administration for tolbutamide after single doses of aqueous solutions and concluded that the routes were equivalent in terms of pharmacokinetics. Values for extrapolated Y-intercepts, plasma half-lives, apparent volume of distribution and metabolic clearance rates were all similar. In this study, since few early measurements of plasma concentration were made, data appeared to follow monoexponential kinetics. Plasma half-life was similar to that described previously (Matin *et al.*, 1974) at 7.7 ± 0.6 h (iv) and 7.6 ± 0.9 h (po) and the apparent volume of distribution was larger (10.1 ± 2.4 L (iv); 9.8 ± 1.7 L (po)), presumably as a result of fitting a monoexponential model. Clearance was calculated to be 15.3 ± 4.3 ml/min (iv) and 15.2 ± 3.5 ml/min (po). The data from these early studies has been confirmed subsequently by Cate *et al.* (1986), where a half-life of 7.8 h, an apparent volume of dimtribution of 11.5 L, a clearance of 17 ml/min and an AUC of 59 mg x min/ml were derived from oral data.

1.2.1.2.5.4 *Use as a Model Compound*

Tolbutamide has potential as a probe of drug metabolism since it is almost completely metabolized in the liver by microsomal enzymes. Metabolism via a single route, oxidation to hydroxytolbutamide, makes it highly sensitive to changes in drug metabolizing activity. It has therefore been used to investigate the effects of inhibitors of drug metabolism (Christensen *et al.*, 1963) and subsequently as a probe of the

effects on drug metabolism of the inhibitor primaquine and the inducer phenobarbitone (Back *et al.*, 1984).

A number of well-known inducers and inhibitors of drug metabolism have been shown to interact with tolbutamide. Elimination is induced by phenobarbitone (Back *et al.*, 1984) and rifampicin (Syvalahti *et al.*, 1974) and inhibited by chloramphenicol (Christensen and Skovsted, 1969), cimetidine (Cate *et al.*, 1986), phenylbutazone (Pond *et al.*, 1978), primaquine (Back *et al.*, 1984), sulphamethizole (Lumholtz *et al.*, 1975), sulphaphenazole (Christensen *et al.*, 1963; Dubach *et al.*, 1966) and sulphinpyrazone (Miners *et al.*, 1982).

1.2.2 *In-vitro* Assays

In-vitro techniques, using liver fractions as enzyme sources, are widely used to identify metabolites and to provide qualitative and quantitative information about possible metabolic pathways. Extrapolation of *in-vitro* data to *in-vivo* drug handling has, however, rarely been predictive. Reasons for this include differences in substrate concentration, availability of co-factors and differences in the *in-vitro* and *in-vivo* rate limiting steps. The rate limiting step for highly cleared drugs, for example, is liver blood flow. Rane *et al.* (1977) were however able to predict the hepatic extraction of a number of compounds covering a wide range (1% to over 90% extraction) by applying quantitative clearance concepts to the *in-vitro* data. This study took into account biological factors which determine organ clearance such as liver weight, blood flow and plasma protein binding (Gillette, 1975; Wilkinson and Shand, 1975). The activity of the hepatic drug metabolizing enzymes was expressed, under first-order conditions, as the ratio of V_{max} and K_m and this in turn may be regarded as the intrinsic clearance of the drug.

Equations 1.2 and 1.5 relate intrinsic clearance, which can be calculated from *in-vitro* data (Rane *et al.*, 1977), to drug clearance *in-*

vivo (section 1.2.1.2.1). Hepatic clearance of other compounds have been predicted in the rat using this concept (Hilliker and Roth, 1980; Huang *et al.*, 1981; Lin *et al.*, 1978; Wiersima and Roth, 1980).

Problems associated with the use of *in-vitro* data to assess the *in-vivo* handling of drugs relate to the source of liver samples and the choice of *in-vitro* and *in-vivo* probes. Human liver samples are usually obtained by biopsy and laparotomy. Since enzyme activity is not thought to be uniform throughout the liver, differences in the location from which liver samples are taken may add to the variability in activity observed. Also liver samples are usually taken only from patients with suspected liver disease, which might, if present, contribute to variability in measured enzyme activity.

The choice of *in-vitro* indicator is often poor in that a number of the classical *in-vitro* substrates tend not to be sensitive to small changes in activity. The most commonly used indicator of *in-vitro* activity, for example, is cytochrome P-450 content. Only small changes can be noted in this parameter however even though large changes occur in the half-life or clearance of *in-vivo* probes such as antipyrine (Vuitton *et al.*, 1981). A number of more specific assays have been developed with specificity for certain forms of cytochrome P-450. The value of these has been investigated in this thesis.

It has been suggested that *in-vitro* estimations should centre on the drug whose elimination is to be predicted *in-vivo*. This approach might be better than the use of an unrelated substrate. Experiments by Boobis *et al.* (1981) using antipyrine in a human study confirmed this idea. There are, however, few other studies of a similar nature owing to the difficulty encountered in obtaining human liver samples.

1.2.2.1 *Assay Techniques*

Many compounds are substrates for the hepatic monooxygenase system and provided that there is a suitable assay procedure any of these may be, and many have been, used to study the drug metabolizing activity within microsomes. Some of these compounds are now rarely used since their assay procedures are tedious, lack sensitivity or selectivity or the compounds are metabolized very slowly or have a complex metabolic routes.

The simple assays for the metabolism of substrates of cytochrome P-450 have previously involved solvent extraction of the metabolite(s) and the use of colorimetric detection methods (e.g ethylmorphine). More sensitive assays measure the fluorescence of the extracted metabolite (e.g benzo(a)pyrene) or the radioactivity of metabolites from a radiolabelled substrate (e.g hexobarbitone). These assays are time intensive and errors can be introduced during the solvent extraction procedures. In order to avoid these problems, direct methods of measurement have been developed. Ullrich and Weber (1972) developed a direct fluorimetric assay to measure the liver microsomal cytochrome P-450 dependent O-dealkylation of 7-ethoxycoumarin to hydroxycoumarin and Burke and Mayer (1974) developed a similar assay which measured the metabolite resorufin produced from O-dealkylation of 7-ethoxyresorufin.

1.2.2.2 *Specificity of Assay Methods*

The discovery that different types of inducing agents differentially induce the metabolism of substrates of the monooxygenase system, promoted studies in this area. It was found that ethoxyresorufin (Burke and Mayer, 1974) metabolism was preferentially induced by 3-methylcholanthrene. This suggested that cytochromes P450I were involved in their metabolism. Aminopyrine-O-demethylase (Nazel, 1971) and dichloronitroanisole-O-demethylase (Hultmark *et al.*, 1979) were found to

be preferentially induced by phenobarbitone, indicating that cytochromes P450IIB are involved in their metabolism. Subsequent studies have shown that 7-methoxycoumarin (Paterson *et al.*, 1984) and pentoxyresorufin (Burke and Mayer, 1983) are preferentially metabolized by phenobarbitone-inducible forms of cytochrome P-450.

The use of substrates specifically metabolized by certain forms of cytochrome P-450 may enable characterization of the induction properties of a compound and may also facilitate measurement and comparison of the ability of different cytochrome P-450 isoenzymes to metabolize drugs (Lecainwasam, 1975; Seymour and Peters, 1977).

1.3 CORRELATIONS BETWEEN *IN-VIVO* AND *IN-VITRO* PARAMETERS OF DRUG METABOLISM

To date investigations of drug metabolizing capacity have produced no significant or very weak correlations of very little predictive value (figure 1.4). This may be due to the use of general probes of drug metabolizing capacity such as cytochrome P-450 content or antipyrine total clearance or half-life which can only reflect gross rather than specific changes in cytochrome P-450. In many of the human studies correlations were only achieved by using a large number of subjects, including diseased patients, or by using normal subjects who were receiving drug therapy. Sotaniemi *et al.* (1977a, 1977b, 1978a, 1978b) and Pirttiaho *et al.* (1978), found correlations between antipyrine half-life or clearance and cytochrome P-450 content in human studies using patients with liver disease. Studies in animals (Statland *et al.*, 1973; Vessell *et al.*, 1973) and normal patients (Davies *et al.*, 1973; Farrell *et al.*, 1979; Sotaniemi *et al.*, 1977a, Vuitton *et al.*, 1981) showed no such correlation however.

Pirttiaho *et al.* (1978) observed, in biopsies from patients free from liver parenchymal changes, that correlations were improved when total

hepatic cytochrome P-450 was used instead of cytochrome P-450 concentration but not when correlation data from biopsies exhibiting parenchymal changes were included. Parenchymal changes such as fat accumulation, increases in fibrotic tissue and inflammatory processes may be associated with liver enlargement and result in changes in microsomal drug metabolizing activity, hepatic drug uptake, blood flow and distribution.

Relationships between antipyrine kinetics and *in-vitro* enzyme activities have also been investigated (figure 1.4). Statland *et al.* (1973) correlated antipyrine half-life with antipyrine hydroxylase activity in rabbits. Davies *et al.* (1973) found no correlation between antipyrine half-life and ethylmorphine N-demethylase activity in humans but Farrell *et al.* (1979) saw a weak correlation in another human study between antipyrine half-life and both ethylmorphine-N-demethylase and aryl hydrocarbon hydroxylase activity. Significant correlations were observed between antipyrine half-life and aniline hydroxylase and ethylmorphine-N-demethylase activities in dogs (Vessell *et al.*, 1973) rabbits (Statland *et al.*, 1973) and marsupials (McManus and Ilett, 1979) (table 1.3).

Human liver biopsy samples have dubious use in the determination of *in-vitro* parameters (section 1.3.2). Apart from the fact that there is usually insufficient information concerning the exposure of the donor to factors which affect the hepatic drug metabolizing enzyme activity, the quantity of tissue is usually insufficient for extensive *in-vitro* investigations with respect to appropriate substrate and co-factor requirements. Animal studies, which can be more easily controlled, have not been fully exploited.

Table 1.3 Relationships observed between *In-vitro* and *In-vivo* Markers of Drug Metabolizing Capacity in Animal Studies

The following correlations were obtained in a study by Vesell *et al.* (1973) using male mongrel dogs (n=12);

	AnOHase (0.35-0.58)	EMdeMease (3.26-8.94)	P-450 (0.38-0.60)	cyt. c reductase (63.5-96.3)	NADPH oxidase (2.6-5.7)
AP t½ (82-194)	-0.78	-0.79	-0.07	-0.21	-0.30
AP MCR (20-72)	0.78	0.82	0.12	0.24	0.30

AP MCR - antipyrine clearance (ml/min)
AnOHase - aniline hydroxylase,
EMdeMease - ethylmorphine N-demethylase.
Values in parentheses indicate the range observed in that particular parameter.

The following correlations were obtained in a study by Miller *et al.* (1978) using male rhesus monkeys (n=6);

	ALE	DHIase	PNAase	BPase
AP t½ (control)	-0.09	-0.27	-0.23	-0.01
AP t½ (PB induced)	-0.59	-0.67	-0.31	-0.75

ALE - aldrin epoxidase
DHIase - dihydroisodrin hydroxylase
PNAase - p-nitroanisole-O-demethylase
BPase - benzo(a)pyrene hydroxylase

Units for enzyme activities - nmol/min/mg protein

Units for cytochrome P-450 - nmol/mg protein

Figure 1.4 Relationships observed between Antipyrine Clearance, and Cytochrome P-450, NADPH Cytochrome c reductase, Aminopyrine demethylase and Aniline hydroxylase Activities in Patients

(from a study by Vuitton *et al.*, 1981)

CYT P450
nmol / mg
0.3
0.5
0.7
20
40
60
r=0.294
NS
CYT c REDUCTASE
nmol /min / mg
50
100
150
20
40
60
r=0.485
p<0.05
ANTIPYRINE CLEARANCE (ml/min)
AMINOPYRINE DEMETHYLASE
μgHCHO/10min/mg
0.5
1
20
40
60
r=0.504
p<0.05
ANILINE HYDROXYLASE
μgPAP/20min/mg
1
2
20
40
60
r=0.563
p<0.02

1.4 AIMS AND OBJECTIVES

The overall aim of this study was to investigate relationships between *in-vivo* and *in-vitro* methods of assessing drug metabolizing ability in the rat. In particular, markers with possible specificity towards particular isoenzymes of cytochrome P-450 were assessed. Both induction and inhibition studies were conducted in order to compare the merits and pitfalls of *in-vivo* and *in-vitro* approaches.

In the induction studies a wide range of drug metabolizing abilities was achieved by using various doses of a cytochromes P450I inducer, β-naphthoflavone, and a cytochromes P450IIB inducer, phenobarbitone. Values for a range of *in-vivo* and *in-vitro* markers of drug metabolizing capacity were determined and inter-relationships investigated. Inter- and intra-individual variability was minimised by using each rat for both the *in-vivo* and the *in-vitro* studies and by simultaneous administration of two of the *in-vivo* model compounds. The potential of a third type of inducer, di-(2-ethylhexyl)-phthalate, for producing a wide range in drug metabolizing capacity was assessed.

In the inhibition studies, the *in-vitro* inhibitory potencies of two imidazoles, ketoconazole and cimetidine, were compared. Ketoconazole inhibition of antipyrine metabolism was determined *in-vivo* in a single dose study and then under steady state conditions. The pharmacokinetics of ketoconazole were studied in order to interpret the single dose inhibition study and to design appropriate dosage regimen for the steady state study.

CHAPTER 2

MATERIALS AND METHODS

2.1 MATERIALS

The suppliers of the chemicals used in the methods described in chapter 2 are indicated below:

Acetonitrile (HPLC grade)	Rathburns Chemicals Ltd., Walkerburn, Scotland
Albumin, Bovine Serum	Sigma Chemical Co.
Aldrin	Chem Service, Pierce & Warner UK Ltd., Chester, England
Aminopyrine	Aldrich Chemical Co.
Ammonium sulphate (GPR)	BDH Chemicals Ltd.
Antipyrine	Sigma Chemical Co.,
(N-methyl-^{14}C)-Antipyrine	The Radiochemical Centre, Amersham, Bucks., England
β-glucuronidase, type HI, *Helix pomatia*	Sigma Chemical Co.
β-hydroxypropyltheophylline	Sigma Chemical Co.
β-naphthoflavone	Sigma chemical Co.
Carbon monoxide	BOC
Chloroform (AR)	Fisons Scientific Apparatus
Chlorpropamide	Gift from Pfizer Ltd., Brooklyn, NY, USA
Cimetidine	Gift from SK&F Labs. Ltd., Herts., England
Clotrimazole	Sigma Chemical Co.
Copper sulphate	BDH Chemicals Ltd.
Corn oil	Mazola, CPC (UK) Ltd., Surrey, England
Cremophor EL	Gift from Pfizer Central Research, Sandwich, Kent, England
Dichlorodimethylsilane	Sigma Chemical Co.
Dieldrin	Chem Service, Pierce & Warner UK Ltd., Chester, England
Diethylether (laboratory chemical)	May & Baker Ltd., Dagenham, Kent, England
Di-(2-ethylhexyl)-phthalate	Aldrich Chemical Co.
Dimethylformamide (SLR)	Fisons Scientific Equipment
Di-sodium hydrogen orthophosphate dodecahydrate (Analar)	BDH Chemicals Ltd.
Ethanol	Fisons Scientific Equipment
Ethanolamine (Technical)	BDH Chemicals Ltd.
7-Ethoxycoumarin (Gold label)	Aldrich Chemical Co.
7-Ethoxyresorufin	Molecular Probes, Oregon, USA
Ethylene diaminetetraacetic acid (EDTA free acid, purified grade)	Sigma Chemical Co.
Folin-Ciocalteu's Phenol reagent (laboratory reagent)	BDH Chemicals Ltd.
Glacial acetic acid (AR)	May and Baker, Dagenham, Kent, England

Heparin, sodium salt, grade II		Sigma Chemical Co.
Hexane (AR)		Fisons Scientific Equipment
Hydrochloric acid (SLR)		Fisons Scientific Equipment
4-Hydroxyantipyrine		Aldrich Chemical Co.
7-Hydroxycoumarin		Aldrich Chemical Co.
3-Hydroxymethylantipyrine	Gift from	Dr.R.Schuppel, Technisch Universtat, Braunschweig, FRG
	and	Dr.M.Lennard, University of Sheffield, England
Ketoconazole	Gift from	Janssen Pharmaceutica, Beerse, Belgium
Lauric acid		Sigma Chemical Co.
^{14}C-Lauric acid		The Radiochemical Centre, Amersham, Bucks., England
Methanol (Technical)		BDH Chemicals Ltd.
Methanol (Analar)		Fisons Scientific Equipment
7-Methoxycoumarin		Sigma Chemical Co.
NADPH, tetrasodium salt, type I		Sigma Chemical Co.
Norantipyrine		Aldrich Chemical Co.
Optiphase MP		Fisons Scientific Equipment
Phenobarbitone Sodium (GPR)		BDH Chemicals Ltd.
Phosphoric acid (SLR)		Fisons Scientific Equipment
Polyethylene Glycol 400 (GPR)		BDH Chemicals Ltd.
Potassium chloride (GPR)		BDH Chemicals Ltd.
Potassium dihydrogen orthophosphate (Analar)		BDH Chemicals Ltd.
Potassium monophosphate (GPR)		BDH Chemicals Ltd.
Propan-2-ol (AR)		Fisons Scientific Equipment
Propylene Glycol (GPR)		BDH Chemicals Ltd.
Resorufin		Eastman Kodak Co., Rochester, N.Y., USA
Soda lime (laboratory reagent)		Fisons Scientific Equipment
Sodium acetate (GPR)		BDH Chemicals Ltd.
Sodium carbonate (GPR)		BDH Chemicals Ltd.
Sodium chloride (GPR)		BDH Chemicals Ltd.
Sodium dithionite (GPR)		BDH Chemicals Ltd.
Sodium hydroxide (laboratory reagent)		BDH Chemicals Ltd.
Sodium metabisulphite (SLR)		Fisons Scientific Apparatus
Sodium potassium tartrate		BDH Chemicals Ltd.
Sucrose (GPR)		BDH Chemicals Ltd.
Sulphuric acid 96% (technical)		Fisons Scientific Equipment
Tolbutamide	Gift from	UpJohn Ltd., West Sussex, England
Toluene (AR)		Fisons Scientific Equipment
Triethylamine (GPR)		BDH Chemicals Ltd.
Tris-(hydroxymethyl)-aminomethane (Analar)		Sigma Chemical Co.
Tris-(hyroxymethyl)-methylammonium chloride (Analar)		BDH Chemicals Ltd.

2.2 ANIMALS

2.2.1 Housing and diet

Male Sprague-Dawley rats (200-300g) were bred and supplied by the University of Manchester Animal Unit. They were housed in opaque plastic boxes (two rats per cage), on a bedding of sawdust and were allowed an unlimited supply of CRM labaura rat diet and fresh drinking water. They were exposed to a 12 h light/dark cycle. Room temperature was maintained between 19 and 21°C.

2.2.2 Pretreatments

2.2.2.1 *Di-(2-ethylhexyl)-phthalate*

Di-(2-ethylhexyl) phthalate (DEHP) was dispersed, unless otherwise stated, in corn oil and administered, intragastrically, using an oral feeder (5ml/Kg, 17.00-18.00 h), once daily for 15 consecutive days.

2.2.2.2 *Phenobarbitone*

Sodium phenobarbitone (PB) was dissolved in normal saline (0.9% w/v) and administered by ip injection (2ml/Kg, 9.00-10.00 h), once daily, for three consecutive days.

2.2.2.3 *β-naphthoflavone*

β-naphthoflavone (β-NF) was finely suspended in corn oil and administered by ip injection (2ml/Kg, 9.00-10.00 h), once daily, for three consecutive days.

2.3 *IN-VITRO* METHODS

2.3.1 Preparation of Microsomes

Rats were killed by cervical dislocation, their livers removed and placed in ice cold potassium chloride solution (10ml; 0.15M). Each liver was weighed, finely chopped with scissors and homogenized in ice cold

SET buffer (0.25M sucrose, 20mM tris-(hydroxymethyl)-aminomethane base; 5.4mM ethylenediamine tetraacetic acid; pH 7.4; 1 volume of liver to 3 volumes SET buffer) with a Potter-Elvehjem homogenizer. Homogenization was standardized to three full strokes. The homogenates were centrifuged (15 min; 15,000g; 4°C; MSE High Speed 21 centrifuge) and the resultant supernatants ultracentrifuged (60 min; 100,000g; 4°C; Sorvall Ultracentrifuge Model OTD 65 B) to produce microsomal pellets which were resuspended in SET buffer (2g wet weight liver/ml) and stored in 1ml eppendorf tubes at -70°C.

2.3.2 Protein Determination

Protein concentrations of prepared microsomes were determined using a modification of the method of Lowry *et al.* (1951). Samples were diluted (1 in 250) with sodium hydroxide solution (0.05M) to give a concentration within the range 100-250µg/ml. Aliquots (0.5ml) of these diluted samples were added to a test tube together with 5ml of Lowry reagent (100ml of sodium carbonate solution, (2% w/v) in sodium hydroxide solution (0.05M); 0.5ml of copper sulphate solution (2% w/v); 0.5ml of sodium potassium tartrate solution (4% w/v), freshly prepared) mixed, and left at room temperature for 15 min. Folin-Ciocalteu's phenol reagent (0.5ml; diluted 1 in 2 with water) was added, mixed immediately and left for a further 30 min. The absorbance (A_{750}) of each sample was measured spectrophotometrically (Pye-Unicam SP8-500), with distilled water in the reference cuvette. A range of protein standards (bovine serum albumin; 0-250µg/ml in 0.05M sodium hydroxide) were assayed concurrently to calibrate the assay. Samples and standards were assayed in triplicate.

2.3.3 Cytochrome P-450 Determination

Microsomal cytochrome P-450 was measured by a modification of the carbon monoxide difference spectral method of Omura and Sato (1964). Samples were diluted (1mg/ml; phosphate buffer, 0.1M, pH 7.4) and aliquots (2ml) pipetted into each of two stoppered 1cm glass cuvettes. A few grains of sodium dithionite were added to each cuvette prior to running baseline spectra (500-400nm; 2nm/second; Pye-Unicam spectrophotometer SP8-500). Difference spectra (490-450nm) were recorded, after bubbling carbon monoxide through the contents of the sample cuvette for 1 min and the absorbance difference was used to calculate cytochrome P-450 content, using an extinction coefficient of 91 mM^{-1} cm^{-1}.

$$\text{Cytochrome P-450 content (nmol mg}^{-1}\text{ microsomal protein)} = \frac{A_{450-490} \times 1000}{91 \times [\text{protein}]}$$

2.3.4 Ethoxycoumarin O-deethylase Activity (ECOD) Determination

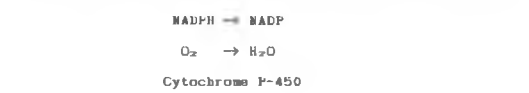
This was a continuous monitoring assay based on the methods of Prough *et al.* (1978) and Ullrich and Weber (1972).

$$\text{7-ethoxycoumarin} \xrightarrow[\text{Cytochrome P 450}]{\text{NADPH} \rightarrow \text{NADP};\ O_2 \rightarrow H_2O} \text{7-hydroxycoumarin} + CH_3CHO$$

Microsomes were diluted (0.5mg/ml) with phosphate buffer (0.1M; pH 7.4), pipetted (2ml) into a quartz fluorimeter cuvette together with 7-ethoxycoumarin (40mM; 10µl; in dimethylformamide) and placed in a temperature controlled cuvette holder (37°C) in a fluorimeter (Perkin Elmer MPF 3a) for 2 min to allow temperature equilibration. A baseline

fluorescence was measured (λ_{ex} 394nm; λ_{em} 456nm; slit width 8nm) and the reaction started by the addition of NADPH (10µl; 20mM; in phosphate buffer (0.1M), pH 7.4). The rate of change in fluorescence was recorded for 2 min and standardized by the addition of 7-hydroxycoumarin (10µl, 1mM in dimethylformamide). Samples were measured in triplicate and results expressed as nmol. of 7-hydroxycoumarin produced per minute per mg of microsomal protein.

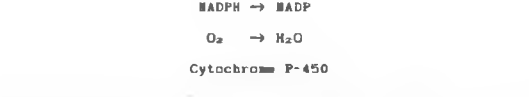
2.3.5 Methoxycoumarin O-demethylase Activity (MCOD) Determination



This assay was identical to that used for the ECOD assay (section 2.3.4.) except that the substrate used was 7-methoxycoumarin (7-MC) (10µl, 40mM in dimethylformamide). Since unpurified 7-MC produced a high background fluorescence which interfered with the assay, the 7-MC was recrystallized from hot methanol.

2.3.6 Ethoxyresorufin-O-deethylase Activity (MROD) Determination

This was a continuous monitoring assay based on the method of Burke and Mayer (1974).



Diluted microsomes (2ml; in phosphate buffer (0.1M), pH 7.4) together with 10µl of 7-ethoxyresorufin (0.2mM; in dimethylformamide) were held for 2 min in a fluorimeter cuvette maintained at 37°C by a temperature controlled cuvette holder in the fluorimeter (Perkin-Elmer MPF 3a). A baseline fluorescence was recorded and then the reaction was started by the addition of NADPH (10µl; 20mM; in phosphate buffer, 0.1M; pH 7.4). Change in fluorescence was measured for about 2 min before the reaction was standardized by addition of resorufin (10µl; 100µM; in ethanol). Samples were assayed in triplicate and results expressed as nmol. of resorufin produced per minute per mg of microsomal protein. Incubates of microsomal preparations from control animals contained 0.25mg/ml (microsomal protein), those from induced animals contained between 0.25 and 0.005mg/ml (microsomal protein).

2.3.7 Aldrin epoxidase Activity (ALN) Determination

This was a batch assay based on the method of Wolff *et al.* (1979).

$$\text{aldrin} \xrightarrow{\text{NADPH} \rightarrow \text{NADP};\ O_2 \rightarrow H_2O;\ \text{Cytochrome P-450}} \text{dieldrin}$$

Prepared microsomes (5ml; in tris buffer (50mM), pH 7.4), together with 5µl of aldrin (100mM, in dimethylformamide), were placed in boiling tubes in a shaking water bath (37°C) for 5 min, to enable temperature equilibration. NADPH (50µl; 20mM; in tris buffer (50mM), pH 7.4) was added to start the reaction, which was incubated for 10 min. Aliquots (1ml) were extracted into hexane (5ml) by rotary mixing (5 min). After centrifugation (2000 rpm; 10min), the aqueous layer was discarded and aliquots (1µl) of the hexane layer were analysed (in duplicate) by gas

chromatography. A calibration curve was constructed by injecting known amounts of dieldrin (0-250pg/µl) onto the gas chromatograph. Incubates of control and β-NF induced microsomes contained a protein concentration of 0.2mg/ml, those of PB induced microsomes contained a protein concentration between 0.2 and 0.05mg/ml. Zero incubation time blanks were run concurrently and subtracted from sample values.

Conditions:-

detector	electron capture
column length	7 feet
support	gas chrom Q
liquid phase	3% OV 255
gas phase	nitrogen
flow rate	30ml/min
oven temperature	235°C
injector temperature	240°C
detector temperature	260°C
gas chromatograph	Hewlett Packard model 5890A

2.3.8 Lauric acid hydroxylase (LAH) Determination

This was a batch assay based on the method of Parker and Orton (1980).

$$\text{Lauric acid} \xrightarrow[\text{Cytochrome P-450}]{\text{NADPH} \rightarrow \text{NADP};\ O_2 \rightarrow H_2O} \text{Hydroxylauric acid}$$

Prepared microsomes (1mg of microsomal protein for controls, 0.5mg for induced microsomes) were placed in a boiling tube together with ^{14}C-lauric acid (100µM; 1µCi; 200µl in tris hydrochloride buffer (50mM),

pH 7.4) and tris hydrochloride buffer (50mM, pH 7.4; to 1.9ml). The boiling tubes were placed in a shaking water bath (37°C) for 5 min to enable temperature equilibration. NADPH (100µl; 20mM; in tris hydrochloride buffer (50mM), pH 7.4) was added to start the reaction which was incubated for 5 min. The reaction was terminated by addition of hydrochloric acid (1M; 0.5ml). The contents of the boiling tubes were transferred to screw-capped test tubes, ether (5ml) added, mixed for 5 min on a rotary mixer, centrifuged to separate the two phases and then the ether layer transferred to clean test tubes. This layer was evaporated down to about 0.5ml, transferred to Eppendorf tubes and then evaporated to dryness. The residues were reconstituted in ether (50µl) and spotted onto a TLC plate (Silica Gel HLF 20cm x 20cm). Plates were developed in a tank equilibrated with hexane : diethylether : glacial acetic acid (60:19:1). The plates were removed, dried and radioactive areas screened using a linear plate analyser (Berthold model LB 277-22 Scanner 11).

2.4 *IN-VIVO* METHODS

2.4.1 $^{14}CO_2$ Exhalation Rate Determination for Antipyrine

Male Sprague-Dawley rats (230-300g) were dosed with (N-methyl-^{14}C-)-antipyrine (ip; 2ml/Kg; 10μCi/Kg; 25mg/Kg) and placed in individual all glass metabolism cages. Using a vacuum pump, air (500-600ml/min) was drawn through a soda lime column, to remove endogenous CO_2, and into the main chamber. The air was then drawn through concentrated sulphuric acid, to remove water vapour, before passing through a CO_2 trapping fluid of ethanolamine:methanol (30ml; 1:4 v/v). Every 20 min this trapping fluid was changed and 0.5ml aliquots taken and added to scintillation fluid (2.5ml; optiphase MP) in individual scintillation vials. Radioactivity in each sample was measured by liquid scintillation counter (LKB 1216 Rackbeta) using an internal quench correction procedure.

Urine was collected for 24 h for radioactivity measurements. After the removal of the rats, each metabolism cage was rinsed and the rinsings added to the urine collected which was made up to a total volume of 100ml with distilled water. Aliquots (100μl) of urine were added to optiphase MP scintillant (2.5ml) in individual scintillation vials in triplicate. These vials were stoppered, shaken, and placed in racks to await counting.

2.4.1.1 *Liquid Scintillation Counting and Calculation of ^{14}Carbon*

2.4.1.1.1 *Urine Samples*

Aliquots (100μl) of diluted urine, together with scintillant (2.5ml; Optiphase MP), were pipetted into individual scintillation vials in triplicate. These vials were stoppered, shaken and placed in racks to await scintillation counting.

To standardize for ^{14}C in the urine, aliquots of the injection solution (100μl) were added to diluted blank urine (100ml in distilled

water) in triplicate. Aliquots (100µl) of this spiked urine, together with scintillant (2.5ml; Optiphase MP), were pipetted into individual scintillation vials in duplicate. The vials were stoppered, shaken and placed in racks to await scintillation counting.

2.4.1.1.2 *Trapping Fluid Samples*

To standardize for ^{14}C in the samples of trapping fluid obtained (section 2.4.1), the radioactivity in the injection solution was determined. Aliquots of the injection solution were added to trapping fluid (30ml, ethanolamine : methanol 1:4 v/v) in triplicate and whirlimixed. Aliquots of the spiked trapping fluid (0.5ml) were pipetted together with scintillant (2.5ml, Optiphase MP) into individual scintillation vials in duplicate. These vials were stoppered, shaken and placed in racks to await scintillation counting.

^{14}C was determined in all of these samples by liquid scintillation counting using an LKB 1216 Rackbeta instrument. Disintegrations per min (DPM) were calculated using the external channels ratio method by reference to a previously generated and stored quench curve. Samples were counted for 10 min with a background of 10 DPM subtracted from the total counts per vial.

2.4.1.2 *Calculation of $^{14}CO_2$ Exhalation Rate (CER)*

$$CER = \% \text{ dose exhaled / min}$$

$$= \frac{DPM_{sample} \div \text{collection time (20 min)} \times 100}{DPM_{inj.\ aliquot} \times \text{inj. vol.} \times 10}$$

2.4.1.3 *Calculation of ^{14}C in Urine*

$$^{14}C \text{ in urine} = \frac{DPM_{urine\ sample}}{DPM_{urine\ standard} \times \text{inj. vol.} \times 10} \times 100$$

2.4.1.4 *Preparation of Injection Solution*

(N-methyl-^{14}C)-antipyrine (250μCi; specific activity 58mCi/mmol) and cold antipyrine (625mg) were dissolved in 50ml of normal saline (0.9% w/v) to give the injection solution (12.5mg/ml; 5μCi/ml).

2.4.1.5 *CER Curve Construction and Use*

The CER values previously calculated (section 2.4.1.1) were plotted as log CER against midpoint time of the collection period. A typical CER-time profile is shown in figure 2.1.

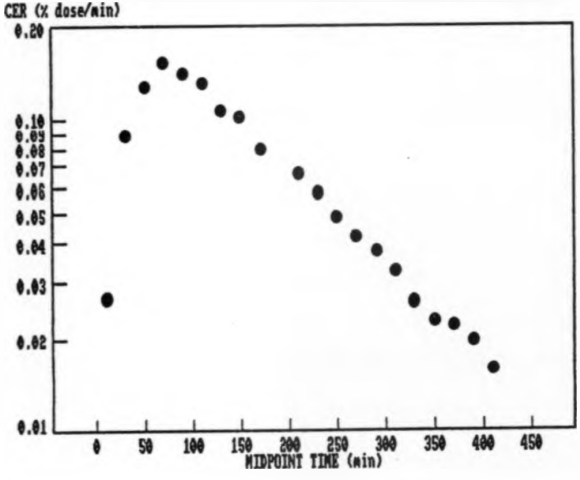
The elimination rate constant (k) was determined, from the slope of the terminal mono-exponential decline phase of the curve, by linear regression. The half-life of antipyrine was determined from ln 2/k. Other parameters determined from the CER data were:

1. maximum CER (CER_{max}) which is the maximum value of CER obtained (% Dose/min)
2. t_{max} which is the time at which the CER_{max} occurs (min)
3. area under the CER-time profile (AUC) (%) which gives an estimate of the percentage of the dose which was exhaled in the breath and therefore the amount of norantipyrine metabolite formed. This was calculated using the trapezoidal approximation by multiplying each CER value by its collection interval and summing the values.

2.4.2 Surgical Procedures

Rats were cannulated prior to experimentation (chapters 4 and 5) under ether anaesthesia. They were returned to individual cages and allowed to recover overnight. During the experiments, the rats were conscious and

Figure 2.1 Typical CER-Time Curve following Administration of (N-methyl ^{14}C) antipyrine


CER (% dose/min)
0.20
0.10
0.09
0.08
0.07
0.06
0.05
0.04
0.03
0.02
0.01
0
50
100
150
200
250
300
350
400
450
MIDPOINT TIME (min)

restrained in cylindrical perspex cages (35cm x 6.5cm i.d.). At all times they were allowed free access to food and water. To prevent blockage of the cannula due to blood clots, each cannula was flushed with heparinised saline (100u/ml) between samples and after drug administration.

2.4.2.1 Carotid artery

A midline incision (5mm) was made through the skin on the ventral side near the neck. Connective tissue was parted to reveal three muscles, the cleidomastoidus, the omohyoideus and the sternohyoides. The sternohyoides and omohyoideus muscles were parted to reveal the trachea and to its right hand side the right common carotid artery. This artery was isolated and carefully separated from the vagus nerve. A ligature was tied at the proximal end of the exposed artery and an artery clip placed distally. A small incision was made in the exposed artery, a cannula (10mm) inserted and secured in place with a ligature. The exposed end of the cannula was drawn under the skin to the dorsal side of the neck of the rat. The incision was sutured and finally the cannula was flushed with 100u/ml heparinised saline. The cannula was prepared by connecting polyethylene tubing (10mm; PE-50; Clay Adams) to silastic tubing (50mm; 0.037in o.d.; Dow Corning) via a metal connector (5mm) made from a hypodermic needle (23G). A small piece of polyethylene tubing (3mm; PE-90; Clay Adams) was slipped over the joint which was then sealed with silastic glue (silicon type A; Dow Corning). A pin was inserted into the exposed end of the silastic tubing as a closure.

2.4.2.2 Jugular vein

A small ventral incision (5mm) was made through the skin over the right jugular vein and the connective tissue parted to expose the jugular vein. A ligature was tied at the proximal end of the isolated vein and a

second ligature was loosely tied distally. A small incision was made in the vein a cannula inserted (20mm) and secured in place with the ligature. The exposed end of the cannula was drawn under the skin to the dorsal side of the neck of the rat, the incision sutured and the cannula flushed with 100u/ml heparinised normal saline. The cannula was prepared as described in section 2.4.2.2 except that the polyethylene tubing section was 20mm long.

2.5 HPLC ASSAYS

2.5.1 HPLC Assay for Antipyrine in Whole Blood

This HPLC assay was a modification of the method of Tounissen *et al.* (1986).

2.5.1.1 *Sample Preparation*

Blood samples were obtained from the carotid artery. Aliquots (100µl) of blood were pipetted into screw-capped test tubes together with a few grains of ammonium sulphate, distilled water (0.5ml) and aminopyrine (internal standard in water, 50µl, 80µg/ml) and extracted into chloroform (5ml) by rotary mixing (10 min). The two phases were separated by centrifugation (2000 rpm, 5 min) and the aqueous layer removed by aspiration. The organic layer was transferred to clean tubes, evaporated to dryness (air, 40°C) and the residue stored in a freezer (-20°C). Samples were reconstituted before analysis in a mobile phase (100µl) of (50:50) acetate buffer (0.2M), pH 5 : acetonitrile. The concentration of antipyrine in the blood samples was determined from a standard curve (0-100mg/L).

2.5.1.2 *HPLC System*

Column	: Spherisorb S5C1 250mm x 4.9mm i.d. (Hichrom Ltd., Reading, England)
Mobile phase	: Acetate buffer (0.2M), pH 5 : acetonitrile (50:50)
Wavelength	: 270nm
Flow rate	: 1.2ml/min
Injection volume	: 20µl
Pump	: Beckman 110B Solvent Delivery Module (HPLC Tech. Ltd., Cheshire, England)
Detector	: Spectroflow 757 absorbance detector (Kratos Analytical Instruments, Ramsey, N.J.)
Recorder	: Phillips Chart Recorder PM 8251 (Phillips Co., Eindhoven, Holland)

2.5.2 HPLC Assay for Theophylline in Whole Blood

This HPLC assay was a modification of the method of Teunissen *et al.* (1986).

2.5.2.1 *Sample Preparation*

Blood samples were obtained from the carotid artery. Aliquots (100µl) of blood were pipetted into screw-capped test tubes together with a few grains of ammonium sulphate, distilled water (0.5ml) and β-hydroxypropyltheophylline (internal standard in ethanol, 50µl, 40µg/ml) and extracted into chloroform : propan-2-ol (95 : 5, 5ml) by rotary mixing (10 min). The two phases were separated by centrifugation (2000 rpm, 5 min) and the aqueous layer removed by aspiration. The organic layer was transferred to clean tubes, evaporated to dryness (air, 40°C) and the residue stored in a freezer (-20°C). Samples were reconstituted in mobile phase (100µl, distilled water : acetonitrile (92:8)) before

analysis. The concentration of theophylline in the blood samples was determined from a standard curve (0-15mg/L).

2.5.2.2 *HPLC System*

Column : Spherisorb S5C1 250mm x 4.9mm i.d.
(Hichrom Ltd., Reading, England)

Mobile phase : distilled water : acetonitrile (92:2)

Wavelength : 274nm

Flow rate : 1.5ml/min

Injection volume : 20µl

Pump, Detector and Recorder see section 2.5.1.2

2.5.3 HPLC Assay for Tolbutamide and Antipyrine in Plasma

This HPLC assay was based on the methods of Nation *et al.* (1978) and Back *et al.* (1984).

2.5.3.1 *Sample Preparation*

Blood samples, obtained from the carotid artery, were centrifuged (2000 rpm, 5 min) to separate plasma from red blood cells. Plasma samples (100µl) were pipetted into screw-capped test tubes, together with chlorpropamide (internal standard in acetonitrile, 20µl, 100µg/ml) and acidified with hydrochloric acid (6M, 4µl) to pH 3. The samples were extracted into chloroform (5ml) by rotary mixing (10 min). The two phases were separated by centrifugation (2000 rpm, 5 min) and the aqueous layer removed by aspiration. The organic layer was transferred to clean tubes, evaporated to dryness (air, 40°C) and the residue stored in a freezer (-20°C). Samples were reconstituted in mobile phase (100µl phosphoric acid solution (1.5% v/v) : acetonitrile (55:45)) before analysis. The concentrations of antipyrine and tolbutamide in plasma

samples were determined from standard curves (0-100mg/L antipyrine and tolbutamide).

2.5.3.2 *HPLC System*

Column : Zorbax ODS 5µm 250mm x 4.6mm i.d.
(Hichrom Ltd., Reading, England)

Mobile phase : Phosphoric acid solution (1.5% v/v) in distilled water : acetonitrile (55:45)

Wavelength : 230nm

Flow rate : 1.5ml/min

Injection volume : 20µl

Pump, Detector and Recorder see section 2.5.1.2

2.5.4 HPLC Assay for Ketoconazole in Plasma

This HPLC assay was developed from the method of Riley and James (1986).

2.5.4.1 *Sample Preparation*

Blood samples obtained from the carotid artery were centrifuged (2000 rpm, 5 min) to separate plasma from red blood cells. Plasma samples (100µl) were pipetted into screw-capped test tubes together with clotrimazole (internal standard in acetonitrile, 10µl, 150µg/ml) and basified with sodium hydroxide (1M, 100µl). The samples were extracted into chloroform (5ml) by rotary mixing (10 min) and the aqueous layer removed by aspiration. The organic layer was transferred to clean tubes, evaporated to dryness (air, 40°C) and the residue stored in a freezer (-20°C). Samples were reconstituted in mobile phase (100µl, potassium monophosphate (0.05M), pH 6 : acetonitrile : triethylamine (50:50:0.05) before analysis. The concentration of ketoconazole in the plasma samples was determined from a standard curve (0-30mg/L).

2.5.4.2 *HPLC System*

Column : Spherisorb S5C1 250mm x 4.9mm i.d.
(Hichrom Ltd., Reading, England)

Mobile phase : potassium phosphate solution (0.05M), pH 6 :
acetonitrile : triethylamine (50:50:0.05)

Wavelength : 230nm

Flow rate : 1.5ml/min

Injection volume : 20µl

Pump, Detector and Recorder see section 2.5.1.2

2.5.5 HPLC Assay of Antipyrine and its Metabolites in Urine

This HPLC assay was essentially the method of Rhodes and Houston (1983).

2.5.5.1 *Sample Preparation*

Urine samples were collected, diluted with distilled water (to 50ml) and stored in a freezer (20°C) until required for analysis.

2.5.5.1.1 *Enzyme Hydrolysis*

Urine samples (0.25ml) were added to screw-capped test tubes together with distilled water (0.25ml) and acetate buffer (0.1M, pH 5, 0.5ml) containing 10,000 units of β-glucuronidase and incubated in a water bath (37°C, 3 h) to hydrolyse conjugates of 3-hydroxymethylantipyrine and 4-hydroxyantipyrine.

2.5.5.1.2 *Acid Hydrolysis*

Urine samples (1.0ml) were added to silanised screw-capped test tubes together with hydrochloric acid (2M, 200µl) and sodium thiosulphate solution (200mg/ml in water, 100µl) and heated in a water bath (100°C, 10 min) to hydrolyse conjugates of norantipyrine. Acetate buffer (0.2M, pH 5.5, 0.5ml) and sodium hydroxide (2M; q.s.) were added to the samples

to adjust the pH to between 6 & 7 (Aqua Scientific pH meter Alpha 500 with microelectrode).

2.5.5.1.3 *Extraction*

Aminopyrine (internal standard 50µg/ml in water, 0.5ml) was added to each hydrolysed sample prior to extraction with chloroform (10ml) by rotary mixing (10 min). The two phases were separated by centrifugation (2000 rpm, 5 min) and the aqueous layer removed by aspiration. The organic layer was transferred to clean tubes (silanised for acid hydrolysed samples), evaporated to dryness (air, 40°C) and the residue stored in a freezer (-20°C). Samples were reconstituted in mobile phase (100µl, acetate buffer (0.2M), pH 5 : methanol (60:40)) before analysis. Concentrations of antipyrine (AP), norantipyrine (NOR), 4-hydroxyantipyrine (4H) and 3-hydroxymethylantipyrine (3H) in urine samples were determined from standard curves (0-50mg/L for AP, NOR and 4H; 0-20mg/L for 3H).

2.5.5.1.4 *Silanisation*

Clean glassware was immersed in a solution of dichlorodimethylsilane (2%) in toluene for 24 h, rinsed first in methanol, then in distilled water and dried.

2.5.5.2 ***HPLC System***

Column	:	Spherisorb S5C1 250mm x 4.9mm i.d. (Hichrom Ltd., Reading, England)
Mobile phase	:	Acetate buffer (0.2M), pH 5 : methanol (60:40)
Wavelength	:	270nm
Flow rate	:	1.0ml/min
Injection volume	:	20µl

Pump, Detector and Recorder see section 2.5.1.2

2.6 DATA ANALYSIS

Since rats used in the invasive *in-vivo* studies were within the weight range 230 to 280g, pharmacokinetic parameters were expressed, where necessary, in terms of a standard rat weight (SRW) of 250g. The elimination half-life (t½) of compounds administered was determined for each animal by least squares linear regression of the log-linear elimination phase of the blood/plasma concentration-time curve constructed from HPLC analysis of samples (0.693/k). Area under the blood/plasma curve (AUC_{∞}) was calculated according to the $\int_0^{\infty}$ trapezoidal rule. Total clearance (CL) was calculated as D/AUC_{∞} in which D is the dose in mg/SRW. The apparent volume of distribution (Vd) was calculated as CL/k.

Where necessary, metabolite formation clearances were calculated from the ratio of the amount of metabolite formed in 4 h (chapter 4) or 10 h (chapter 5) to the $AUC_{4\,h}$ (chapter 4) or $AUC_{10\,h}$ (chapter 5). Mean data is tabulated with standard deviations in parentheses unless otherwise specified.

CHAPTER 3

INVESTIGATION OF THE DRUG METABOLISM MODULATING POTENTIAL OF DI-(2-ETHYLHEXYL)-PHTHALATE

3.1 INTRODUCTION

The use of phthalate esters as vehicles, for pesticides, in insect repellent and within cosmetic products, has resulted in significant levels contaminating the environment. Various phthalate esters have been detected in rivers and fish (Mayer *et al.*, 1972), soil (Ogner and Schnitzer, 1970), foodstuffs (Tomita *et al.*, 1977) and in domestic animals (Taborsky, 1967; Nazir *et al.*, 1971). The phthalate ester, di-(2-ethylhexyl)-phthalate (DEHP), is used as a plasticizer in the manufacture of a wide range of products but in particular polyvinyl chloride (PVC). Such materials are often used as containers for medicinal products. In this respect DEHP has been shown to leach from containers into stored blood (Thomas and Thomas, 1984) and has been detected in recipient's blood and in human tissues (Jaeger and Rubin, 1972). In view of the chronic exposure of man to DEHP, a number of workers have investigated the potential toxicological hazard associated with their use.

3.1.1 Toxicity Studies

Acute animal toxicity studies have demonstrated large amounts of DEHP to be well tolerated in the rat (oral LD_{50} values of 26 to greater than 34g/Kg) and rabbit (oral LD_{50} value of 34g/Kg) (Gray *et al.*, 1977; Fassett, 1967; Hodge, 1943; Shaffer *et al.*, 1945). Similar LD_{50} values were found in the rat following acute ip administration (greater than 23.8g/Kg); (Hodge, 1943) but on chronic administration these values were reduced.

Exposure of laboratory animals to high doses of DEHP results in a variety of biological effects which include liver enlargement (Brown *et*

al., 1978; Lake *et al.*, 1975; Nikonorow *et al.*, 1973), testicular atrophy (Cater *et al.*, 1977; Foster *et al.*, 1980; Gray *et al.*, 1977; Lake *et al.*, 1975, 1984a), proliferation of peroxisomes and endoplasmic reticulum (especially in rodent species) (Lake *et al.*, 1984b; Reddy and Krishnakantha, 1975), hypolipidaemia (Ball *et al.*, 1978), changes in plasma proteins (Hinton *et al.*, 1985), liver tumorigenesis (Douglas and Hartwell, 1981; Kluwe *et al.*, 1982) and alteration of various markers of xenobiotic metabolism (Lake *et al.*, 1975; Tanaka *et al.*, 1978).

3.1.1.1 *Liver Tumorigenesis*

DEHP has been reported to be a peroxisome proliferator and a weak hepatocarcinogen both in mice and female rats (Kluwe *et al.*, 1982; N.T.P., 1982). The mechanism for its hepatocarcinogenicity is, however, unknown. Reddy *et al.* (1979, 1980, 1982, 1983, 1986) and Goel *et al.* (1986) have suggested that the increased number of peroxisomes and activity of peroxisomal enzymes associated with peroxisomal proliferation might be directly related to the carcinogenic process, whereby excess production of H_2O_2 through increased fatty-acyl Co-A oxidase activity, causes diffusion of active oxygen species from the peroxisomes into the cytoplasm and nucleus (figures 3.1 and 3.2). Such active oxygen species and associated free radicals have been generally implicated as initiators and promoters of the carcinogenic process. Direct studies on the effects of DEHP as a promoter (Popp *et al.*, 1985) or initiator (Garvey *et al.*, 1987) of carcinogenesis have, however, proved to be negative in that they have failed to detect genotoxic effects attributable to DEHP (Butterworth *et al.*, 1984; Von Daniken *et al.*, 1984; Zeiger *et al.*, 1982). Generally compounds which act as initiators of carcinogenesis are genotoxic.

It is doubtful whether rodent carcinogenicity data can be extrapolated to assess human risk, although Turnbull and Rodricks (1985)

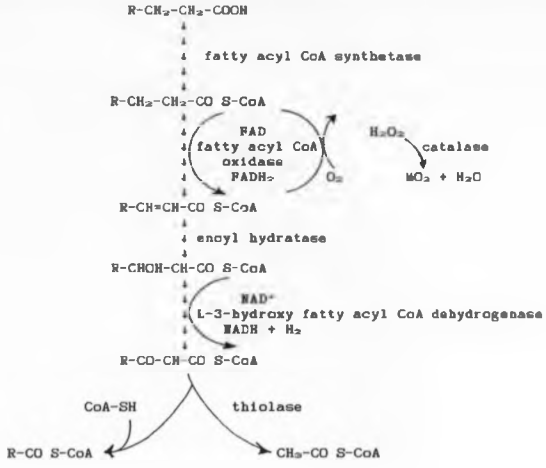


Figure 3.1 Pathway of Peroxisomal β-oxidation of Fatty Acids

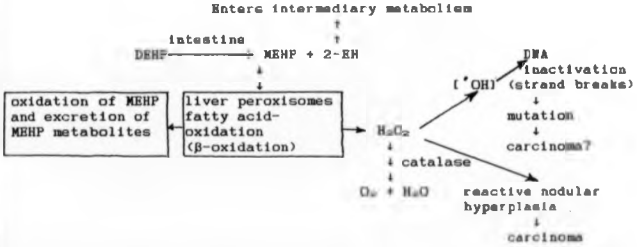


Figure 3.2 Schematic Diagram of the Peroxisomal Proliferation Hypothesis

At high doses of DEHP it is proposed that excess H_2O_2 or other oxygen species are produced in excessive amounts because catalase production does not increase as rapidly as peroxide production.

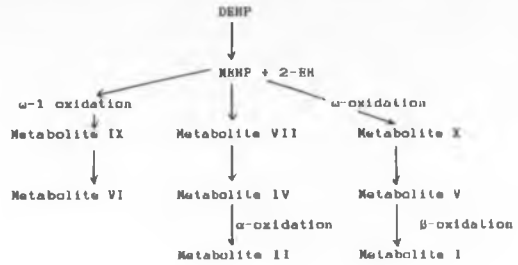
have attempted to do so using available data on toxicity, genotoxicity, metabolism, pharmacokinetics and physiological and biochemical effects of DEHP. Indeed care must be taken in interpreting all of these data. Particularly, Barber *et al.* (1985) examined the standard methods used to determine mutagenicity and found that the extraction procedures employed were inadequate for the quantitative recovery of the metabolites of DEHP.

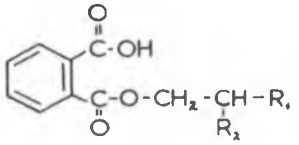
3.1.1.2. *Testicular Atrophy*

High dose administration of DEHP resulted in testicular and accessory sex gland atrophy (Gray *et al.*, 1977; Oishi and Hiraga, 1979), increased testicular testosterone concentration (Oishi and Hiraga, 1980) and reduced testicular and anterior prostrate zinc concentrations (Curto and Thomas, 1982; Oishi and Hiraga, 1980). It is well documented that dietary zinc deficiency is often associated with the occurrence of testicular atrophy (Millar *et al.*, 1958, 1960; Prasad, 1966, 1976; Underwood, 1971) and that the effect can normally be counteracted by administration of zinc (Chandra *et al.*, 1975; Gunn *et al.*, 1961; Parizek, 1957). Testicular atrophy associated with DEHP could not be prevented by coadministration of zinc (Oishi and Hiraga, 1983). A study by Oishi (1985) showed that testicular atrophy induced by DEHP is of limited reversibility even 45 days from cessation of treatment.

3.1.2. Metabolism of DEHP

Figure 3.3 depicts the urinary metabolites of DEHP and MEHP which have been isolated and the pathways by which they may be produced. Following ingestion by the rat, DEHP is substantially hydrolysed to the monoester MEHP and 2-ethylhexanol (2-EH) by non-specific lipases of the pancreatic juice (Albro and Thomas, 1973; Daniel and Bratt, 1974; Lake *et al.*, 1977; Rowland, 1974; White *et al.*, 1980). Subsequently the absorbed MEHP





Metabolite	R_1	R_2
I	$-CH_2-COOH$	$-CH_2-CH_3$
II	$-(CH_2)_3-CH_3$	$-COOH$
III	$-(CH_2)_2-COOH$	$-CH_2-CH_3$
IV	$-(CH_2)_3-CH_3$	$-CH_2-COOH$
V	$-(CH_2)_2-COOH$	$-CH_2-CH_3$
VI	$-(CH_2)_2-CO-CH_3$	$-CH_2-CH_3$
VII	$-(CH_2)_4-CH_3$	$-CH_2-CH_2OH$
VIII	$-CH_2-CHOH-CH_2-CH_3$	$-CH_2-CH_3$
IX	$-(CH_2)_2-CHOH-CH_3$	$-CH_2-CH_3$
X	$-(CH_2)_4-CH_2OH$	$-CH_2-CH_3$
(MEHP) XI	$-(CH_2)_4-CH_3$	$-CH_2-CH_3$
XII	$-CH_2-CHOH-CH_2-COOH$	$-CH_2-CH_3$

Figure 3.3 Major Pathways of Metabolism of DEHP in Rats

The pathways illustrated are inferred from knowledge of the structures of the urinary metabolites shown, based on the work of Albro et al. 1981. Metabolites are numbered according to Albro's convention.

is metabolized through formation, in the liver, of metabolites derived from the ω- and (ω-1)-oxidation products of the alkyl moiety of MEHP (Albro *et al.*, 1973; Albro and Moore, 1974; Chu *et al.*, 1978; Daniel and Bratt, 1974; Williams and Blanchfield, 1974). These reactions and the analogous oxidative reactions on the absorbed 2-EH are catalysed by the NADPH-dependent hepatic microsomal mixed function oxidase enzymes (Lake *et al.*, 1975).

Several minor metabolites have been identified (Albro *et al.*, 1983). In rats and mice, metabolite V (a product of ω-oxidation) may undergo β-oxidation with loss of a 2-carbon unit to yield metabolite I. Metabolite I has not been detected in humans and is only a minor metabolite of primates (Albro *et al.*, 1983). Lower levels of metabolite I (Turnbull and Rudricks, 1985) in primates rather than rodents have been attributed to less extensive peroxisome proliferation in the primate following DEHP administration.

Metabolism of MEHP is extensive and varies quantitatively and qualitatively between species (Albro *et al.*, 1982a, 1982b). Such species differences may partially explain the observed lack of peroxisomal proliferation in man following exposure to DEHP (Elcombe *et al.*, 1983; Lake *et al.*, 1984a), since MEHP metabolites are the proximate peroxisome proliferators in the rat (Mitchell *et al.*, 1985).

3.1.3 Pharmacokinetics of DEHP and MEHP

Pollack *et al.* (1985) administered DEHP by three different routes to rats and examined the kinetics of DEHP and its primary metabolite MEHP. DEHP doses administered were 2g/Kg, 4g/Kg and 100mg/Kg and produced similar blood concentrations following po, ip and intra-arterial (ia) administration respectively. Following a single ia injection a large apparent volume of distribution (5390 ml/Kg) and a medium clearance (21.5 ml/min/Kg) were observed for DEHP. The systemic bioavailability of

DEHP was low following a single po (13.6%) or ip (5.2%) dose and differences, for each route of administration, were observed for the formation of MEHP. After oral administration, blood concentrations of MEHP were higher than those of DEHP (AUC ratio = 7) but lower for iv and ip administration (AUC ratio < 0.4). This was because 80% of the oral dose underwent mono-de-esterification as opposed to 1% by the iv and ip administration routes. Such data highlight the importance of presystemic DEHP hydrolysis to MEHP in the gut. Poor bioavailability of DEHP by the ip route must be attributed to slow and/or incomplete absorption, since only a small proportion of the ip dose was mono-de-esterified. As predicted by the single dose data there was no significant accumulation of DEHP or its metabolite MEHP in the blood following seven daily po or ip doses of DEHP. An apparent decrease in the rate and/or extent of DEHP absorption from the peritoneal cavity was observed following multiple ip administration but not for po absorption.

MEHP (50mg/Kg) administered iv (Pollack *et al.*, 1985) has a smaller volume of distribution (201 ml/Kg) and a lower clearance (11.5 ml/min/Kg) than DEHP. The terminal half-life for the administered MEHP was shorter than that value derived from the metabolism of DEHP following either po (6.9 h) or ip (9.6 h) administration, the terminal half-life of DEHP being 15 h. The bioavailability of MEHP by the po (60%) and ip (84%) routes was higher than that of DEHP. Lower availability of MEHP given po rather than ip may result from first pass metabolism of the monoester in the gut wall or within the gut lumen. Alternatively it may result from incomplete absorption from the gut. Sjoberg *et al.* (1986) found the mean elimination half-life of MEHP, following oral administration, to be significantly shorter in animals given repetitive doses than in those given a single dose. No statistically significant difference was, however, observed between the respective mean AUC values.

Sjoberg *et al.* (1985) also examined disposition of various doses of infused DEHP. Concentrations were at all times higher than those of MEHP and the concentrations of MEHP were much higher than those of the other investigated metabolites. Parallel decreases in the plasma concentrations of DEHP, MEHP and the ω and (ω-1)-oxidized metabolites indicated that the elimination of DEHP was the rate limiting step in the disposition of the metabolites. This was supported by the observation that the clearance of MEHP was lower than that of DEHP. Non-linear increases in the AUC values of DEHP and MEHP indicated saturation in the formation, as well as the elimination, of the potentially toxic metabolite MEHP. Whilst the same species and strain were used in the studies of Pollack *et al.* (1985) and Sjoberg *et al.* (1985), differences were observed with respect to the apparent volume of distribution, half-life and clearance of DEHP.

3.1.4 Induction of Microsomal Parameters by DEHP

DEHP has been reported to interact with, and modify, biological responses to parathion (Srivastava *et al.*, 1976), barbiturates, methaqualone (Daniel and Bratt, 1974; Rubin and Jaeger, 1973; Seth *et al.*, 1977) and carbon tetrachloride (Seth *et al.*, 1979). Biochemical investigations, into the hepatic effects of these compounds, have shown that they might affect mitochondrial function (Lake *et al.*, 1975; Srivastava *et al.*, 1978; Takahashi, 1977), produce peroxisome proliferation (Moody and Reddy, 1978) and induce peroxisomal marker enzymes such as cyanide-insensitive palmitoyl-CoA oxidation and carnitine acetyltransferase (Lake *et al.*, 1984a, 1984c). Peroxisome proliferators, such as DEHP and clofibrate, have been reported to markedly enhance the microsomal oxidation of fatty acids (Okita and Chance, 1984; Orton and Parker, 1982) but to have a lesser effect (both induction and inhibition) on other microsomal enzyme activities (Agarwal

et al., 1982a, 1982b; Aitio and Parkki, 1978; Lake *et al.*, 1975; Pollack and Shan, 1984; Srivastava *et al.*, 1978). This is in addition to increasing peroxisomal enzymes activities such as the enzymes of the peroxisomal fatty acid β-oxidation cycle (Lazarow and DeDuve, 1976). This has led to the suggestion that these compounds may induce a novel form of cytochrome P-450. Indeed, Gibson and co-workers (Gibson *et al.*, 1982; Tamburini *et al.*, 1984) and Hardwick *et al.* (1987) have isolated a form of cytochrome P-450 (P450IV) from the livers of clofibrate-treated rats which has a high specificity for the ω- and (ω-1)-hydroxylation of lauric acid, but correspondingly low specificity towards typical mixed function substrates. It is likely therefore that the notable liver enlargement, increased cytochrome P-450 content and increased endoplasmic reticulum (Daniel and Bratt, 1974; Lake *et al.*, 1975) in DEHP treated rats are associated with peroxisomal effects and induction of the microsomal fatty acid ω hydroxylation system.

3.2 THE EFFECT OF DEHP ADMINISTRATION ON *IN-VITRO* AND *IN-VIVO* PROBES OF DRUG METABOLIZING CAPACITY

3.2.1 Introduction

Inducing agents have been classified into two main groups, polycyclic aromatic hydrocarbon-like (PAH-like) or phenobarbitone-like. It would appear, however, from the literature (Hardwick *et al.*, 1987) that a third group, incorporating peroxisome proliferators, exists. One of the intentions of this project was to examine *in-vitro/in-vivo* correlations through induction of a wide range of drug metabolizing enzyme activities using rats and different types of inducing agents. An investigation by Pollack and Shen (1984) indicated that pretreatment of rats with DEHP significantly increased the clearance of antipyrine, one of the markers of drug metabolizing capacity for these intended correlation studies. Since DEHP appears to be a suitable novel inducer compound representing the third class, preliminary studies were conducted to determine the effects of DEHP dose and its length of administration on the rat microsomal oxidation system.

3.2.2 Experimental Design

Male Sprague-Dawley rats (200 to 300g) were given daily oral administration (at 17.00-18.00 h) of either corn oil (n=3, 5ml/Kg) or DEHP in corn oil (n=3, 2g/Kg, 5ml/Kg) for 15 days. Two days before the first administration of corn oil/DEHP, (N-methyl-^{14}C)-antipyrine was administered ip to each rat (25mg/Kg; 10μCi/Kg; 2ml/Kg) and the animals were placed in all glass metabolism cages. Antipyrine $^{14}CO_2$ exhalation rates (CER) were determined as described in section 2.4.1. The antipyrine breath test was repeated on all rats at day 15 prior to the final corn oil/DEHP dose. The rats were kept in metabolism cages for 24 h and urine collections made. Radioactivity levels in urine samples were determined to ensure good recovery. On day 16, rats were killed by

cervical dislocation and their livers removed for preparation of microsomes (section 2.3.1). For each microsome preparation, microsomal protein determination (section 2.3.2), cytochrome P-450 content (section 2.3.3), ECOD (section 2.3.4) and EROD activities (section 2.3.6) were measured. Radioactivity in the 24 h urine collections was measured and used as an indicator of metabolite excretion (section 2.4.1).

3.2.3 Results and Discussion

3.2.3.1 *Effect of DEHP administration on antipyrine ^{14}CER Data*

Typical CER-time curves, generated from antipyrine breath test data, are shown in figure 3.4 both before and after 14 days administration of DEHP in corn oil (2g/Kg, 5ml/Kg). The control curves generated, before and after corn oil administration, were superimposable and showed no significant differences in any of the CER parameters (table 3.1). Treatment with DEHP, however, decreased the antipyrine half-life (t½), by 20%, the time to peak CER (tmax), by 40% and area under the curve (AUC), by 24% but produced no change in CERmax. Whilst there was clearly an induction of antipyrine metabolism, since the AUC was decreased, then the data suggested that less was metabolized via the N-demethylation pathway. The induction of antipyrine was, therefore, selective towards the hydroxylation pathways of antipyrine.

3.2.3.2 *Effect of DEHP administration on in-vitro parameters*

DEHP produced hepatomegaly (liver weight/100g body weight increased by 74%), increased cytochrome P-450 content (42%) and ECOD activity (83%) but had little or no effect upon EROD activity (table 3.2). Whilst the hepatomegaly produced by DEHP (74%) was much greater than that observed for high doses of PB (20%) or β-NF (20%), the increases in cytochrome P-450 content (42%) were smaller than that produced by PB (250%) or β-NF (250%). Similarly for ECOD the increase in activity, induced by DEHP,

Figure 3.4 Typical (N-methyl-^{14}C)-antipyrine CER-Time Curves obtained before and after 14 Daily Oral Doses of DEHP (2g/Kg)

■ - Before treatment

● - After treatment

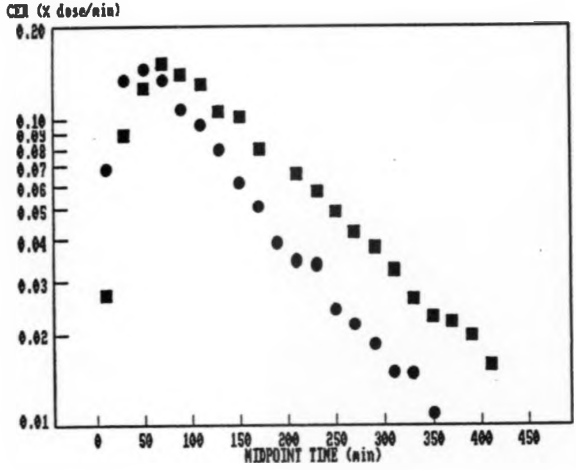

CEI (% dose/min)
0.20
0.10
0.09
0.08
0.07
0.06
0.05
0.04
0.03
0.02
0.01
0
50
100
150
200
250
300
350
400
450
MIDPOINT TIME (min)

Table 3.1 Effect of DEHP or Corn Oil Treatment on (N-methyl ^{14}C)-antipyrine CHR Parameters

Parameter	Treatment	Day 0 of Treatment	Day 15 of Treatment
$t_{1/2}$	*CO	127.8 (3.5)	129.3 (0.3)
$t_{1/2}$	*DEHP + CO	114.1 (22.3)	*90.9 (11.8)
CHR_{max}	CO	0.135 (0.007)	0.143 (0.024)
CHR_{max}	DEHP + CO	0.149 (0.025)	0.146 (0.015)
t_{max}	CO	54 (4)	40 (10)
t_{max}	DEHP + CO	67 (15)	*40 (10)
AUC	CO	26.4 (0.8)	27.3 (0.7)
AUC	DEHP + CO	27.8 (0.8)	*21.1 (0.7)

*CO - corn oil (5ml/Kg)
*DEHP + CO - DEHP in corn oil (2g/Kg)
Significantly different from pretreatment values;
* $p < 0.1$; ** $p < 0.01$; *** $p < 0.005$
Determined using the paired t test.

Table 3.2 Effect of DEHP Treatment on *In-vitro* Microsomal Data

Parameter	Treatment	
	Corn oil (5ml/Kg)	DEHP (2g/Kg)
LW/BW	3.5 (0.3)	*5.9 (0.2)
P-450	0.33 (0.09)	*0.48 (0.05)
ECOD	0.60 (0.17)	*1.10 (0.11)
EROD	0.12 (0.03)	0.14 (0.01)

Significantly different from pretreatment values;
* $p < 0.1$; ** $p < 0.05$; *** $p < 0.005$
Determined using the t-test

was by far the smallest (83%) when compared to that for PB (5 fold) and β-NF (14 fold). Unlike DEHP, both PB and β-NF induced EROD activity (0%, 60% and 100 fold respectively) (tables 4.4 and 4.6).

3.2.4 Conclusions

The observed reduction in half-life, following DEHP administration, indicated induction of antipyrine metabolism. The degree of effect on antipyrine elimination was much less than that observed with other classical inducers, which suggested that the cytochrome P-450 isoenzymes induced by DEHP have a low affinity for antipyrine as a substrate. Increases occurred in two of the microsomal parameters, cytochrome P-450 and ECOD activity, following DEHP treatment, but again the classical inducers are known to produce much greater increases in activity (Taylor *et al.*, 1985).

3.3 EFFECT OF DEHP DOSE AND LENGTH OF DOSING PERIOD ON *IN-VIVO* AND *IN-VITRO* PROBES OF DRUG METABOLIZING CAPACITY

3.3.1 Introduction

The aim of these studies was to produce a wide range in drug metabolizing capacity in rats to enable *in-vivo/in vitro* correlations. Thus the inducing potential of three different doses of DEHP were investigated. Preliminary studies employing DEHP (2g/Kg; section 3.2) had produced induction of antipyrine metabolism but the dosing interval, of 14 days, was unwieldy. The study was repeated here using, in addition, two lower doses of DEHP and carrying out several breath tests, at frequent intervals, during the fifteen days oral dosing. Thus it was hoped to eventually be able to reduce the dosing period for subsequent studies.

3.3.2 Experimental Design

Male Sprague-Dawley rats (200 to 300g) were orally dosed with either corn-oil (5ml/Kg), or DEHP in corn oil (0.5, 1 or 2g/Kg as 5ml/Kg), on each of fifteen days at 17.00 - 18.00 h. Three rats were used for each treatment group. On the morning of the first dose and also on days three, seven, ten and fourteen, the animals were placed in all glass metabowls, were given (N-methyl-^{14}C)-antipyrine (ip) and exhaled $^{14}CO_2$ collected (section 2.4.1). On each occasion, 24 h urine collections were made to ensure good recovery of radioactivity. After the fifth breath test, rats were given a 15th dose (17.00 to 18.00 h). The following day, rats were removed from the metabowls, killed by cervical dislocation and their livers removed for preparation of microsomes (2.3.1). Various *in vitro* assays were carried out on prepared microsomes (sections 2.3.2, 2.3.3, 2.3.4 and 2.3.6). Urine (24 h) was collected and the levels of radioactivity measured (section 2.4.1).

3.3.3 Results and Discussion

3.3.3.1 *Effect of DEHP on Antipyrine CER Parameters – Dose and Dosing Interval Dependencies*

CER-time profiles, produced before and after treatment with corn oil or DEHP (0.5 and 1g/Kg), were essentially superimposable, indicating that these treatments had little effect on antipyrine disposition. A significant change in profile was observed for the highest dose of DEHP (2g/Kg) only (figure 3.5). The change occurred gradually, reached a maximum at seven days and was maintained up to day 14. As in the previous study there was a significant decrease in half-life with DEHP (2g/Kg; figure 3.6) and no change in CER_{max} (table 3.3). Differences were seen, however, between the two studies for this DEHP dose. There was an increase instead of a decrease in t_{max} and no effect instead of a decrease in AUC (table 3.3).

3.3.3.2 *Effect of Dose of DEHP Administered on In-vitro Parameters*

Mean data (table 3.4) showed dose dependant increases in relative liver weight, cytochrome P-450 content and ECOD activity but slight decreases in ERCOD activity. Whilst maximal hepatomegaly was observed for the two highest doses of DEHP (1g/Kg and 2g/Kg), effects upon cytochrome P-450 content and ECOD activity were greater for the 0.5g/Kg and 2g/Kg dose than the 1g/Kg dose (table 3.4).

Figure 3.5 Typical (N-methyl-^{14}C)-antipyrine ^{14}CHR-Time Curves generated before and After 3, 7, 10 and 14 Daily Oral Doses of DEHP (2g/Kg)

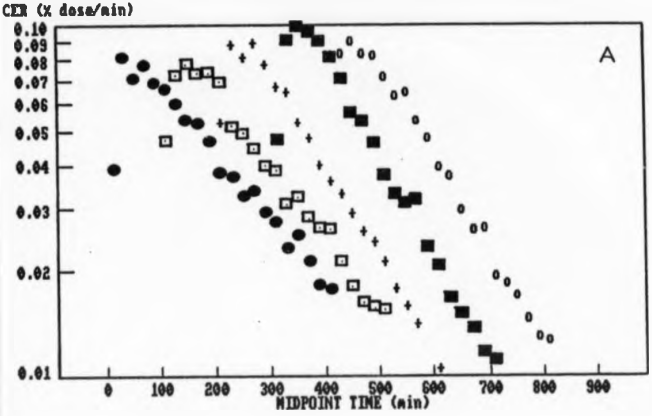
In this figure the various data sets, derived from one rat, have been displaced in 100min increments for clarity

- ● - Before treatment
- □ - After 3 daily doses
- + - After 7 daily doses
- ■ - After 10 daily doses
- ○ - After 14 daily doses

Figure 3.6 Pictorial Representation of the Change (%) in Half-life of Antipyrine with Dose and Dosing Interval of DEHP

Mean data is shown;

- ● - corn oil (5 ml/Kg) (n=3)
- ■ - DEHP in corn oil (0.5g/Kg) (n=3)
- ◆ - DEHP in corn oil (1 g/Kg) (n=3)
- ✕ - DEHP in corn oil (2 g/Kg) (n=3)


CER (x dose/min)
0.10
0.09
0.08
0.07
0.06
0.05
0.04
0.03
0.02
0.01
A
0
100
200
300
400
500
600
700
800
900
MIDPOINT TIME (min)

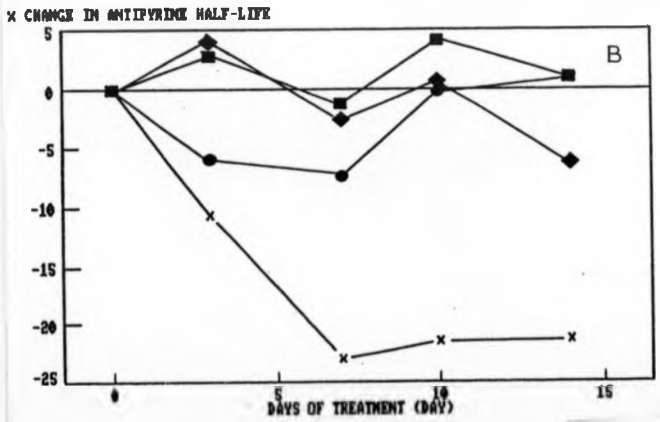

% CHANGE IN ANTIPYRINE HALF-LIFE
5
0
-5
-10
-15
-20
-25
B
0
5
10
15
DAYS OF TREATMENT (DAY)

Table 3.3 Effect of Varying Dose and Dosing Interval of Administered DEHP on (N-methyl-^{14}C)-antipyrine CHR Parameters

Parameters	Treatment	Days of treatment				
		0	3	7	10	14
$t_{1/2}$	CO	121.8 (8.7)	115.3 (23.3)	114.1 (17.2)	122.0 (21.6)	123.1 (17.2)
$t_{1/2}$	DEHP (0.5g/Kg)	111.7 (9.4)	114.5 (6.3)	110.0 (4.3)	*116.5 (12.4)	112.7 (7.5)
$t_{1/2}$	DEHP (1 g/Kg)	117.4 (7.2)	122.1 (13.9)	114.3 (10.9)	117.2 (4.5)	*110.2 (9.3)
$t_{1/2}$	DEHP (2 g/Kg)	140.1 (18.5)	*125.1 (15.7)	*107.3 (8.7)	*108.5 (4.9)	*116.9 (7.1)
CHR_{max}	CO	0.103 (0.008)	0.104 (0.014)	0.097 (0.014)	0.102 (0.012)	0.106 (0.010)
CHR_{max}	DEHP (0.5g/Kg)	0.128 (0.009)	*0.120 (0.010)	*0.117 (0.005)	*0.100 (0.010)	*0.115 (0.010)
CHR_{max}	DEHP (1 g/Kg)	0.125 (0.008)	0.113 (0.016)	0.104 (0.011)	*0.093 (0.009)	*0.107 (0.007)
CHR_{max}	DEHP (2 g/Kg)	0.089 (0.020)	0.084 (0.006)	0.085 (0.007)	0.089 (0.009)	0.078 (0.014)
t_{max}	CO	45 (18)	58 (6)	53 (3)	50 (5)	60 (5)
t_{max}	DEHP (0.5g/Kg)	57 (17)	50 (0)	48 (3)	57 (7)	49 (6)
t_{max}	DEHP (1 g/Kg)	43 (11)	54 (5)	55 (16)	60 (10)	*57 (2)
t_{max}	DEHP (2 g/Kg)	43 (11)	*60 (2)	50 (0)	57 (7)	63 (6)
AUC	CO	17.7 (0.7)	*19.4 (1.0)	16.5 (2.0)	21.0 (0.5)	20.9 (0.6)
AUC	DEHP (0.5g/Kg)	22.9 (0.1)	*20.4 (0.7)	*19.9 (0.7)	*18.4 (0.0)	*19.4 (0.2)
AUC	DEHP (1 g/Kg)	22.8 (0.5)	*19.8 (0.7)	20.2 (2.8)	*17.6 (0.5)	*18.5 (1.0)
AUC	DEHP (2 g/Kg)	16.6 (1.8)	15.9 (0.2)	14.8 (2.4)	16.4 (1.5)	16.4 (0.0)

Significantly different from pretreatment values:
* $p < 0.1$
* $p < 0.05$
* $p < 0.01$
* $p < 0.005$
Determined using the paired t test

Table 3.4 Effect of Varying Dose of DEHP Administered on In-vitro Studies

Parameter	Treatment			
	Corn oil	DEHP 0.5g/Kg	DEHP 1g/Kg	DEHP 2g/Kg
LV/BV	3.4 (0.2)	*4.5 (0.2)	*5.4 (0.2)	*5.5 (0.3)
P-450	0.36 (0.08)	b0.48 (0.04)	0.43 (0.08)	b0.52 (0.02)
ECOD	0.72 (0.07)	0.88 (0.13)	0.82 (0.19)	*1.02 (0.10)
EROD	0.15 (0.04)	0.11 (0.01)	*0.09 (0.02)	b0.08 (0.02)

Significantly different from the corn oil control:
* p < 0.05 level
b p < 0.01 level
c p < 0.001 level
Determined using one way ANOVA

3.3.4 Conclusions

Antipyrine half-life decreased only with the highest dose of DEHP (2g/Kg). During the period of treatment, the decrease in half-life reached a minimum at day seven and was then maintained. Since no decrease in half-life was observed following 14 daily doses of 1g/Kg of DEHP, this is not equivalent to seven daily doses of 2g/Kg. These CER results confirmed that cytochromes P450IV$_A$ do not have an important role in the metabolism of antipyrine.

In-vitro, dose-related changes were observed in increases in liver weight and cytochrome P-450. The microsomal enzymes differed in their response to DEHP treatment. ECOD activity increased and EROD activity decreased. ECOD is a general substrate which is inducible by both PB and β-NF. EROD is a more specific assay for β-NF induction.

3.4 EFFECT OF CONCURRENT ADMINISTRATION OF DEHP AND CORN OIL ON *IN-VIVO* AND *IN-VITRO* PARAMETERS OF DRUG METABOLISM

3.4.1 Introduction

The previous two studies (Sections 3.2 and 3.3) showed anomalous *in-vitro* results associated with DEHP administration. In these studies the standard diluent for DEHP was corn oil. Since DEHP is an oily liquid then in order to maintain a constant dose volume, different proportions of DEHP corn oil were used. A possibility existed, therefore, that the rats were responding to corn oil induced metabolism, rather than to the DEHP. Such possibilities were investigated by monitoring various markers of drug metabolizing capacity following administration of DEHP alone (2g/Kg, 2ml/Kg), DEHP with corn oil (2g/Kg, 2ml/Kg and 3ml/Kg respectively), corn oil alone (3ml/Kg) and sodium chloride 0.9% w/v (3ml/Kg).

3.4.2 Experimental Design

Male Sprague-Dawley rats (200 to 300g) were orally dosed either with saline (0.9% w/v, 3ml/Kg), corn oil (3ml/Kg) or DEHP alone (2g/Kg, 2ml/Kg) and in corn oil (2g/Kg; 2ml/Kg and 3ml/Kg). Four rats were included in each group giving a total of 16 rats, dosed at between 17.00 and 18.00 h each day. The animals were subjected to an antipyrine breath test (section 2.4.1) before and after the treatments. The saline treated group were not included in the *in-vivo* study but were treated (4 rats sodium chloride 0.9% w/v, 3ml/kg) concurrently with the other three groups. Following the second breath test all rats received a 15th dose and on the following day were removed from the metabowls, killed by cervical dislocation and their livers removed for preparation of microsomes (section 2.3.1). *In-vitro* parameters were measured (section 2.3.2, 2.3.3, 2.3.4, and 2.3.6) and radioactivity in the urine samples (24 h collection) was determined (section 2.4.1).

3.4.3 Results and Discussion

3.4.3.1 *Effects of DEHP and Corn oil on CER Parameters*

CER_{max} and t_{max} were unaffected by any of the three treatments. Half-life and AUC were unaffected by corn oil treatment. Similar decreases in half-life were observed for the two DEHP groups but a larger decrease in AUC was observed for the group receiving both corn oil and DEHP (table 3.5).

Table 3.5 Effects of administering Corn oil, and DEHP with or without Corn oil, on (N-methyl-^{14}C)-antipyrine CER Parameters

Parameter	Treatment	Day 0 of Treatment	Day 15 of Treatment
t½	CO	122.8 (16.2)	122.4 (12.8)
t½	DEHP (alone)	135.4 (25.5)	*112.5 (8.7)
t½	DEHP (+ CO)	124.8 (17.0)	*106.7 (29.6)
CER_{max}	CO	0.111 (0.010)	0.107 (0.010)
CER_{max}	DEHP (alone)	0.107 (0.010)	0.102 (0.010)
CER_{max}	DEHP (+ CO)	0.110 (0.022)	0.085 (0.020)
t_{max}	CO	49 (8)	51 (8)
t_{max}	DEHP (alone)	47 (6)	51 (2)
t_{max}	DEHP (+ CO)	55 (6)	61 (10)
AUC	CO	21.7 (0.5)	*20.5 (0.8)
AUC	DEHP (alone)	20.7 (0.7)	*17.2 (1.2)
AUC	DEHP (+ CO)	20.8 (0.8)	*14.7 (0.6)

Significantly different from pretreatment values:
* $p < 0.1$
** $p < 0.005$
*** $p < 0.0005$
Determined using the paired t-test

3.4.3.2 *Effects of DEHP and Corn oil on In-vitro Parameters*

Hepatomegaly was greater for the DEHP with corn oil group than the DEHP alone group. This suggests that corn oil in the diet might potentiate the liver changes produced by agents such as DEHP, whilst being unable to initiate such changes by itself. No significant effects were observed for cytochrome P-450 in any instance. ECOD activity increased in the order saline 0.9% < corn oil < DEHP < DEHP + corn oil. The ECOD activity of the DEHP alone group was twice that of the saline group but essentially similar to that of the corn oil group. The ECOD activity of the DEHP + corn oil group was 1.5 times bigger than the corn oil group and the DEHP group but three times bigger than the saline group. The order of increase in the EROD activity was saline 0.9% < DEHP < DEHP + corn oil < corn oil. Changes produced were small and were only statistically different for corn oil when compared with each of the other 3 groups (table 3.6).

Table 3.6 Effects of Administering Saline, Corn oil, and DEHP with or without Corn oil on *In-vitro* Studies

Parameter	Treatment			
	Saline	Corn oil	DEHP alone	DEHP + CO
LW/BW	4.0 (0.3)	*3.3 (0.3)	*4.9 (0.4)	*5.8 (0.7)
P-450	0.30 (0.02)	0.39 (0.05)	*0.40 (0.08)	0.38 (0.06)
ECOD	0.35 (0.01)	*0.65 (0.09)	*0.70 (0.04)	*0.95 (0.15)
EROD	0.04 (0.01)	*0.08 (0.01)	0.05 (0.01)	0.06 (0.02)
LAH	3.42 (0.52)	*5.67 (0.94)	*13.82 (3.50)	*17.83 (3.39)

Significantly different from saline control values:
^ $p < 0.05$ value; ^ $p < 0.01$ value
* $p < 0.001$ value
Determined using one way ANOVA

Lauric acid hydroxylase activity was substantially increased in the DEHP treated groups. The increase in this parameter followed the same trend observed with ECOD activity. This indicated that corn oil had a slight inductive effect on this microsomal enzyme specific for P450IV forms of cytochrome P-450. Clearly, although small changes were observed in other parameters, DEHP treatment was having an inductive effect.

3.4.4 Conclusions

These results confirmed the small but significant effect of DEHP on antipyrine half-life. Both with and without corn oil diluent, the DEHP produced a decrease in half-life. In addition, a decrease in AUC was observed as previously reported in section 3.2. This indicates that the importance of N-demethylation of antipyrine is reduced in the DEHP-induced rat.

The *in-vitro* data confirms and expands on the earlier studies. From the ECOD data, it was seen that corn oil produced a similar increase as DEHP alone when compared to the saline group, but DEHP with corn oil produced the largest increase and it was observed to be an additive effect since the activity doubled. The decrease in EROD data originally observed for DEHP (section 3.3) was confirmed in this study but was not apparent when the two DEHP groups were compared with the saline group. This indicated that corn oil induced EROD activity. The corn oil effects are only exhibited *in vitro* in these studies. The antipyrine breath test did not pick up changes caused by corn oil administration. These results show the importance of not only testing the vehicle but also ensuring that the volume of vehicle used is the same in both control and test animals. In addition, they reinforce the difference in sensitivity between microsomal and *in-vivo* tests.

3.5 FINAL DISCUSSION

3.5.1 *In-vivo* studies

In all three studies, no change in CER parameters were observed for the animals pretreated with corn oil. Rats pretreated with DEHP in corn oil (2g/Kg), however, showed a significant decrease in half-life with no change in CER_{max}. In two studies (section 3.2 and 3.4), AUC values were decreased but in the third (section 3.3) no change in AUC for rats given DEHP in corn oil (2g/Kg) was recorded. When data from all three studies was averaged however, a significant decrease in AUC was observed. A different trend was observed in each of the studies for the parameter t_{max} for rats given DEHP in corn oil (2g/Kg). In study 1 (section 3.2) t_{max} decreased, in study 2 (section 3.3) t_{max} increased and in study 3 (section 3.4) t_{max} was unchanged. When the data was averaged for t_{max}, no significant difference could be demonstrated in this parameter.

Study 2 (section 3.3) showed that corn oil pretreatment and the lower doses of DEHP in corn oil (0.5 and 1g/Kg), produced no effects on ^{14}CER parameters. The decrease in half-life observed for the highest dose of DEHP (2g/Kg) occurred gradually and reached a plateau value after seven days treatment. This was maintained until day 15 of treatment.

In study 3 (section 3.4), the decreases, in antipyrine half-life produced by DEHP alone and with corn oil, were similar but the decrease, in AUC, was larger in the group receiving DEHP in corn oil. When data from the three studies was averaged, however, no difference could be demonstrated between these two groups.

These data indicate that DEHP alone and with corn oil is a poor inducer of antipyrine metabolism at the doses studied. The degree of effect was much less than that observed for classical inducers of drug metabolism and suggests that the isoenzymes of cytochrome P-450, induced by DEHP, have low affinity for the substrate antipyrine. Pollack and Shen's (1984) study demonstrated DEHP to have a more marked effect on

antipyrine clearance (3.9 -> 7.8ml/min/Kg) and half-life (165 -> 67.2min) than was observed in the present work. They also observed little or no effect upon urinary recovery of the antipyrine metabolites. Although in the present investigations no analysis of the urine was made for metabolites, a decrease in AUC for the CER-time profiles was observed. This indicated a reduction in the amount of antipyrine being metabolized to norantipyrine. The unusually low recovery of norantipyrine (3% for the corn oil group and 2% for the DEHP treated group) observed by Pollack and Shen (1984) suggested that losses may have occurred during either the hydrolysis or extraction stages. This could explain, in part, the differences between their results and the present studies, since normally a recovery for this metabolite of about 20% would be expected. Indeed a small effect on antipyrine metabolism is more in keeping with the small changes observed in the *in-vitro* parameters observed here and also by Pollack and Shen (1984). Pollack and Shen (1984) also suggested from their data that the induction of antipyrine metabolism by DEHP resembled the induction produced by phenobarbitone. This conclusion cannot be drawn from the CER data presented here since phenobarbitone induction causes a large decrease in half-life (small decrease with DEHP), an increase in CER_{max} (no change with DEHP), a decrease in t_{max} (no change with DEHP) and a decrease in AUC (decrease with DEHP) (Rhodes and Houston, 1983). In a similar fashion, DEHP induction does not resemble that observed with β-NF for which large decreases in half-life, increases in CER_{max} and increases in t_{max} and AUC are observed. Since the liver was greatly enlarged by DEHP it is likely that the effects observed are due to this rather than to actual increases in specific activity of the drug metabolizing enzymes. Surprisingly 14 daily doses of 1g/Kg DEHP did not produce a similar effect to seven daily doses of 2g/Kg. The effects of DEHP are not therefore cumulative.

Table 3.7 (N-methyl-^{14}C)-antipyrine CER Data Averaged from the Three DEHP Studies

Parameter	Treatment	Day 0 of Treatment	Day 15 of Treatment
$t_{1/2}$	CO (3ml/Kg)	122.8 (16.2)	122.4 (12.8)
$t_{1/2}$	CO (5ml/Kg)	124.8 (6.8)	126.2 (11.4)
$t_{1/2}$	DEHP + CO (0.5g/Kg)	111.7 (9.4)	112.7 (7.5)
$t_{1/2}$	DEHP + CO (1 g/Kg)	117.4 (7.2)	[b]110.2 (9.3)
$t_{1/2}$	DEHP + CO (2 g/Kg)	126.2 (19.9)	[a]104.4 (20.7)
$t_{1/2}$	DEHP alone (2 g/Kg)	135.4 (25.5)	[a]112.5 (8.7)
CER_{max}	CO (3ml/Kg)	0.111 (0.010)	0.107 (0.010)
CER_{max}	CO (5ml/Kg)	0.119 (0.019)	0.124 (0.026)
CER_{max}	DEHP + CO (0.5g/Kg)	0.127 (0.009)	[b]0.115 (0.010)
CER_{max}	DEHP + CO (1 g/Kg)	0.125 (0.008)	[a]0.107 (0.007)
CER_{max}	DEHP + CO (2 g/Kg)	0.115 (0.032)	0.103 (0.033)
CER_{max}	DEHP alone (2 g/Kg)	0.107 (0.010)	0.102 (0.010)
t_{max}	CO (3ml/Kg)	49 (8)	51 (2)
t_{max}	CO (5ml/Kg)	48 (12)	52 (12)
t_{max}	DEHP + CO (0.5g/Kg)	57 (17)	49 (6)
t_{max}	DEHP + CO (1 g/Kg)	43 (11)	[a]57 (2)
t_{max}	DEHP + CO (2 g/Kg)	54 (13)	53 (12)
t_{max}	DEHP alone (2 g/Kg)	47 (8)	51 (8)

continued overleaf

Table 3.7 cont. (N-methyl-^{14}C)-antipyrine CHR Data Averaged from the Three DEHP Studies

Parameter	Treatment	Day 0 of Treatment	Day 15 of Treatment
AUC	CO (3ml/Kg)	21.7 (0.5)	$^{=}$20.5 (0.8)
AUC	CO (5ml/Kg)	22.0 (4.9)	24.1 (3.7)
AUC	DEHP + CO (0.5g/Kg)	22.7 (0.3)	$^{=}$19.4 (0.2)
AUC	DEHP + CO (1 g/Kg)	22.7 (0.3)	*18.5 (1.1)
AUC	DEHP + CO (2 g/Kg)	21.8 (4.7)	$^{=}$17.0 (3.0)
AUC	DEHP alone (2 g/Kg)	20.7 (0.7)	$^{=}$17.2 (1.2)

Significantly different from pretreatment values;
^{a} $p < 0.1$; ^{b} $p < 0.05$;
^{c} $p < 0.01$; ^{d} $p < 0.005$
Determined using the paired t-test

3.5.2 *In-vitro* Studies

The dose dependent increases (Table 3.8) in liver size observed here following DEHP treatment have also been reported by a number of other workers (Gray *et al.*, 1977; Lake *et al.*, 1975, 1984a). Such liver effects are produced by a wide variety of substances and are frequently associated with increases in the activity of the hepatic microsomal drug-metabolizing enzymes (Conney, 1967; Schulte-Hermann, 1974). In such circumstances enlargement has been regarded as an adaptive physiological response of the liver to increased metabolic demand (Golberg, 1966). DEHP, however, only had a small effect on the metabolism of substrates normally used for *in-vitro* determination of drug metabolizing capacity. Various studies have suggested that such observations are indicative of incipient hepatotoxicity (Crampton *et al.*, 1977; Grasso *et al.*, 1974).

Similarly, the livers of DEHP treated animals showed a marked proliferation of peroxisomes. This effect is also thought to be an indication of hepatocarcinogenicity (Reddy *et al.*, 1980; Lalwani *et al.*, 1981). No assays were carried out to establish induction of peroxisomal enzymes since studies by Lake *et al.* (1984c, 1986) have established that there are strong correlations between lauric acid hydroxylase activity and both cyanide-insensitive palmitoyl-Co A oxidase (r=0.97) and carnitine acetyltransferase (r=0.98), in the rat. Lauric acid hydroxylase activity was significantly increased in these studies and palmitoyl-Co A oxidase is a specific peroxisomal marker and carnitine acetyltransferase is located in peroxisomal, mitochondrial and microsomal fractions.

DEHP administration produced only small increases in cytochrome P-450 content and ECOD activity. Both of these markers reflect changes in cytochrome P-450 isoenzymes. A slight decrease in EROD activity, a marker of PAH induction was observed and the results of the second study suggested that these decreased at the expense of increases in other forms of cytochrome P-450. The values for P-450 and ECOD activity after administration of the 1g/Kg DEHP dose were lower than that expected if the changes were simply dose related. The results of the third study provide a possible reason for this. Corn oil administration had no effect on liver weight but increased cytochrome P-450 content, ECOD and EROD activity relative to saline administration. The increase in liver weight, P-450 content and ECOD activity was greater when DEHP was administered with corn oil than when administered alone. The effects of DEHP and corn oil on P-450 content and ECOD activity were additive and the effects on EROD activity could be totally explained by the effects of the corn oil diluent and were dose dependent.

The only microsomal parameter to be greatly affected by the treatments was lauric acid hydroxylase activity. This is a microsomal

Table 3.8 *In-vitro* Microsomal Parameters averaged from the three DEHP Studies

Parameter	Treatment						
	Saline	Corn oil		DEHP + corn oil			DEHP alone
	3ml/Kg	3ml/Kg	5ml/Kg	0.5g/Kg	1g/Kg	2g/Kg	2g/Kg
LW/BW	4.0 (0.3)	b3.3 (0.3)	a3.5 (0.2)	4.5 (0.2)	c5.4 (0.2)	c5.8 (0.5)	b4.9 (0.4)
P-450	0.30 (0.02)	0.39 (0.05)	0.34 (0.08)	b0.48 (0.04)	a0.43 (0.08)	b0.45 (0.08)	a0.41 (0.08)
ECOD	0.35 (0.01)	b0.65 (0.09)	a0.66 (0.13)	a0.88 (0.13)	a0.82 (0.19)	c1.02 (0.13)	c0.70 (0.04)
EROD	0.04 (0.01)	a0.08 (0.01)	a0.13 (0.04)	b0.11 (0.01)	a0.09 (0.02)	b0.09 (0.04)	0.05 (0.01)
LAH	3.42 (0.52)	a5.67 (0.94)	----	----	----	a17.83 (3.39)	a13.82 (3.50)

Significantly different from saline controls:
a $p < 0.05$
b $p < 0.01$
c $p < 0.001$
Determined using one way ANOVA

parameter specific for P450IV inducers. In this instance the corn oil once again exerted additive effects with the order of increasing activity being saline < corn oil < DEHP alone < DEHP + corn oil.

Although it is often assumed that corn oil exerts little effect on microsomal enzyme activity, with weak inducers, coadministration with corn oil may produce anomalies. Observation of such an anomaly with DEHP, led to the work carried out in the third study. In the literature, free fatty acids have a number of influences upon mixed function oxidation. A chronic fat-free diet decreases cytochrome P-450 content and rates of microsomal oxidation of several substrates (Horred and Wade, 1972). Depletion of dietary fat also decreases the ability of PB to induce cytochrome P-450 (Marshall and McLean, 1971). Reconstituted

mixed function oxidase (mfo) systems require phosphatidylcholine for maximum activity (Coon *et al.*, 1976). Free fatty acids cause a shift from low to high spin states in the haem iron (Gibson *et al.*, 1980). At high concentrations, (3.5mM) free fatty acids act as detergents and inhibit drug metabolism (Augustine and Fouts, 1969). A study by Wattenberg *et al.* (1962), has reported that in the rat, feeding a low fat diet results in a markedly lower activity of benzo(a)pyrene hydroxylase, a microsomal enzyme. Since fatty acids have multiple effects on both NADPH-generating processes and the microsomal P-450 system it is difficult to predict their net action on mixed function oxidase activity (Danis *et al.*, 1985). Previous workers have disregarded these effects by administering the same dose volume rather than the same vehicle volume in studies with DEHP or do not indicate clearly the volume of vehicle administered and so this must be taken into consideration when interpreting the DEHP data in the literature.

CHAPTER 4

USE OF INDUCED ANIMALS TO INVESTIGATE THE RELATIONSHIPS BETWEEN *IN-VITRO* AND *IN-VIVO* MARKERS OF DRUG METABOLIZING CAPACITY

4.1 INTRODUCTION

The factors which influence the ability of an individual to metabolize administered drugs and methods of assessing these have been extensively studied (Conney and Kappas, 1985; Breimer, 1983; Vessell, 1979; Vessell and Penno, 1983; Vessell, 1984). Inter-individual variability, which may confound such studies, can be minimised by using each individual as his or her own control. The pharmacokinetics of a model compound such as antipyrine may thus be determined in the individual both before and after applying just one environmental change (Vessell, 1979).

The use of model compounds in order to predict the pharmacokinetics of another drug substance has been of limited success, mainly as a consequence of the multiplicity of the drug metabolizing enzyme system. The actual complement of drug metabolizing enzymes found in an individual at any particular time, depends upon a large number of factors which interact with each other and the genetic background. In order for the pharmacokinetics of one compound to be useful for predicting the pharmacokinetics of another, therefore, similar isoenzymes of cytochrome P-450 must be involved in their metabolism. For example, although hexobarbitone and heptobarbitone clearances were found to correlate strongly in the rat (r=0.96) (Van der Graaff *et al.*, 1983a) a similar study with hexobarbitone and antipyrine produced poor correlations (Van der Graaff *et al.*, 1983b).

An alternative approach has been to use metabolite formation clearance data. The hypothesis that the different pathways of antipyrine metabolism may be mediated by different isoenzymes has been the subject of a number of investigations (table 1.2). Indeed although hexobarbitone

clearance did not correlate with antipyrine clearance, good correlations were achieved when the formation clearance of 3-hydroxymethylantipyrine was used (Van der Graaff *et al.*, 1983b).

The intention of these studies was to investigate possible correlations in rats between and within various *in-vivo* and *in-vitro* marker compounds having specificity for different forms of cytochrome P-450. Since there is relatively little variability in drug metabolism in untreated rats, two types of inducer, a typical 3-MC-type inducing agent, β-naphthoflavone (β-NF) (Boobis *et al.*, 1976), and phenobarbitone (PB), were administered at various dose levels in order to achieve a wide range in drug metabolizing enzyme activities, whilst keeping all other factors, which might affect drug metabolism, constant.

4.2 BACKGROUND TO THE INDUCTION PROCESS

A wide range of therapeutic drugs are known to produce enzyme induction in animals (Conney, 1967; Remmering, 1968). For many years inducers of cytochrome P-450 were thought to fall into two major categories; those which act like 3-methylcholanthrene (3-MC) and those which act like phenobarbitone (PB). The use and development of sophisticated techniques for the characterization of cytochromes P-450, showed that there were more species of P-450 than those induced by "PB-type", or "3-MC-type" compounds. Other studies have shown that many inducers do not act like either 3-MC or PB (Nebert *et al.*, 1981). On the basis of substrate specificity, amino acid composition, NH_2-terminal sequence, and immunoreactivity (Goldstein, 1984; Brunnick *et al.*, 1984) cytochromes P-450 have now been classified either as constitutive or induced forms. The latter have been subdivided, so far, into five groups according to the types of chemicals which cause their induction (table 4.1) (Goldstein, 1984; Davies, 1984; Nebert and Gonzalez, 1985; Nebert *et al.*, 1981). There are still, however, known inducing agents which

Table 4.1 Categories of Compounds Inducing Cytochromes P-450 in Laboratory Animals

Type of Inducer	Examples of Compounds within group
3-Methylcholanthrene	Polycyclic aromatic hydrocarbons - benzo(a)pyrene, benz(a)anthracene, TCDD, β-NF
Phenobarbitone	Most barbiturates, anticonvulsants
Ethanol	
Clofibrate	Peroxisome proliferators: - DEHP
Pregnenolone-16-α-carbonitrile	Glucocorticoids: - dexamethasone

produce a response which does not fit into any of these categories.

Many compounds induce both phase I and phase II enzymes. Rat liver microsomal epoxide hydrolase activity is induced by PB, polycyclic aromatic hydrocarbons (PAHs) of the 3-MC-type, pregnenolone-16-α-carbonitrile (PCN), antioxidants and polychlorinated biphenyls (PCB) (Bentley and Oesch, 1982). UDP-glucuronosyl-transferase activity is induced in rat liver by both PB and 3-MC (Owens, 1977). Cytosolic glutathione-S-transferase activity in rat liver is induced by PB (Hales and Reims, 1977), 3-MC (Hales and Reims, 1977; Pickett *et al.*, 1982), trans-stilbene oxide and 2-acetylaminofluorene (DePierre *et al.*, 1984). Certain chemicals such as butylhydroxyanisole (Benson *et al.*, 1979) cause dramatic induction of glutathione-S-transferases and/or epoxide hydrolase whilst having relatively little effect on cytochromes P-450. These highly induced levels of Phase II enzymes may be important in protecting the animals from reactive metabolites formed from potentially toxic or carcinogenic substances (Wattenberg, 1985).

4.2.1 Effects of Inducing Agents on the Liver

Both PB and 3-MC produce increases in liver size and synthesis of hepatic proteins. Only PB however, for which the onset of induction is much slower than for 3-MC, produces increases in microsomal protein synthesis and proliferation of smooth endoplasmic reticulum (Conney *et al.*, 1956; Conney and Gilman, 1963; Arcos *et al.*, 1961). Other studies have shown that compounds from both of these groups markedly increase the quantity of other components of the electron transport system functioning in drug metabolism (Remmer and Merker, 1965).

4.2.2 Mechanism of Induction of Drug-Metabolizing Enzymes

It is now established that induction of several cytochrome P-450 species requires *de-novo* protein (Nebert and Gonzalez, 1985; Nebert *et al.*, 1981; Adesnick and Atchison, 1986). Increased catalytic activity of P-450 mediated monooxygenases is a consequence of increased synthesis of cytochrome P-450 apoproteins rather than being due to enzyme stabilization or to post-transcriptional "activation" of latent enzyme. Regulation of cytochrome P-450 induction is thus primarily at the level of gene transcription.

In-vivo treatment of animals with compounds which induce cytochromes P-450, leads to a rapid increase in transcription of mRNAs that code for specific induced P-450 apoproteins (Nebert and Gonzalez, 1985; Nebert *et al.*, 1981; Adesnick and Atchison, 1986) For example, treatment of rats with PB produces, within 3 h, measurable increases in mRNA specific for the PB-induced forms of P-450; within 16 h the level of specific mRNAs in PB-treated rats may be 25 to 100 times greater than the level of the same mRNAs in untreated rats (Adesnick and Atchison, 1986). Immunoprecipitation of P-450 using antibodies to the P-450 apoprotein indicates that increases in P-450 apoprotein are quantitatively comparable to the levels of induced mRNA. Specific forms of P-450 may

therefore be dramatically increased (25- to 100-fold) whilst the overall cytochrome P-450 pool generally only increases 2- to 3-fold.

Regulation of Phase II enzymes also appears to be predominantly at the transcription level. In rat liver, induction of glutathione S-transferase B by 3-MC can be totally accounted for by the increased rates of synthesis of its mRNA (Pickett *et al.*, 1982). PB induction increases the rate of transcription of epoxide hydrolase genes by 4- to 9-fold in rat liver and this results in increased epoxide hydrolase enzymatic activity (Hardwick *et al.*, 1983).

4.2.2.1 *Mechanism by which 3-MC-type Compounds Initiate the Induction Process*

Although it is clear that the increased enzyme activity is due to increased transcription, far less is known about how the cell initially recognises the presence of enzyme inducers and how this information is transmitted to transcriptional sites. Only in the case of induction of cytochromes P450I by 3-MC-type compounds is the overall induction mechanism relatively well understood.

3-MC-type inducers are hydrophobic polynuclear aromatic molecules that either are rigidly planar (3-MC, halogenated dibenz-*p*-dioxins, halogenated dibenzofurans) or can be rotated to a coplanar configuration (PCBs other than those with ortho substituents). It is thought that they enter cells by simple diffusion through the plasma membrane (Nebert and Bausserman, 1971) and then bind to a soluble intracellular protein called the Ah receptor (Ah=aromatic hydrocarbon) (Nebert *et al*, 1981; Poland *et al.*, 1979; Okey *et al.*, 1979; Carlstedt-Duke *et al.*, 1978). After the chemical inducer binds to the Ah receptor, the inducer-receptor complex undergoes a temperature-dependent "activation" process which converts the complex to a form which is able to enter the nuclear compartment and then to bind with high affinity to specific gene regions

(Eisen *et al.*, 1982; Okey *et al.*, 1980). The specific gene region at which the inducer-Ah receptor complex acts has been tentatively identified in cultures of hepatoma cells by molecular-genetic techniques (Jones *et al.*, 1985).

Tritiated tetrachlorodibenzo-*p*-dioxin (TCDD) has been used to identify and characterize the Ah receptor (Poland *et al.*, 1979; Okey *et al.*, 1979; Carlstedt-Duke *et al.*, 1978; Poland and Knutson, 1982; Safe *et al.*, 1985; Eisen *et al.*, 1982). The TCDD-Ah receptor complex appears to act at a specific gene region approximately 1200 to 1500 base pairs upstream from the promoter gene region which controls the P450I structural gene (Jones *et al.*, 1985). The effect of binding of the TCDD-Ah receptor complex to this gene region is to increase the rate of transcription of the P450I structural gene, perhaps by releasing the promoter from inhibition (Jones *et al.*, 1985). The P450I mRNA thus synthesized is then translated into P450I apoprotein, which, after addition of the haem prosthetic group, is incorporated into cell membranes where the enzyme becomes catalytically active.

4.2.2.1.1 *Evidence Linking the Ah Receptor to Regulation of Cytochrome P450I Induction*

It has clearly been shown that the Ah receptor satisfies all the necessary criteria to be considered a true receptor which mediates induction of cytochrome P450I by 3-MC-type chemicals. The evidence linking the Ah receptor to regulation of cytochrome P450I induction is a strong quantitative structure-activity relationship. The affinity with which most compounds bind to the Ah receptor correlates well with the *in-vivo* potency of the compounds as inducers of cytochrome P450I in laboratory animals. This relationship has been established for several subcategories of the 3-MC-type of P450I inducers including halogenated

congeners of dibenzo-*p*-dioxins and dibenzofurans (Poland and Knutson, 1982) and polychlorinated biphenyls (PCBs) (Safe *et al.*, 1985).

Nebert and colleagues (Nebert *et al.*, 1981; Safe *et al.*, 1985) found that certain strains of inbred mice were "non-responsive" to induction of cytochrome P450I when treated with 3-MC. "Non-responsive" mice have normal structural genes which code for the cytochrome P450I apoprotein itself (Nebert *et al.*, 1981; Safe *et al.*, 1985). The heritable "non-response" to 3-MC appears to be a mutation in the regulatory gene which codes for the Ah receptor protein. As a result of this mutation, the receptor protein has a reduced affinity for 3-MC-type inducers. The Ah receptor, initially discovered in rodents, now has been shown to exist in many mammalian species (Denison and Wilkinson, 1985) including humans (Roberts *et al.*, 1985) and primates (Okey *et al.*, 1983).

4.2.2.1.2 *Ah Receptor-Cytosolic or Nuclear?*

When cells are fractionated by standard techniques of homogenization and differential centrifugation, the unoccupied form of Ah receptor appears to be a cytosolic component (Okey *et al.*, 1979, 1980); however, a recent study suggests that the Ah receptor, prior to occupancy by the inducing chemical, is localized within the nuclear compartment from where it is released during cell fractionation (Whitlock and Galeazzi, 1984). Although the initial subcellular localization of the unoccupied form of the Ah receptor has not yet been decided, the final site at which the receptor regulates enzyme induction is agreed to be within the nucleus.

4.2.2.2 ***Mechanism of Induction by other Agents***

The mechanism of induction of drug-metabolizing enzymes for other categories of inducers is not yet clear. It has been postulated that a specific receptor exists which mediates the inductive effect of PB-type compounds, but the lack of strong structure-activity relationships for

PB-type inducers and the lack of a specific PB-type ligand with high affinity for the postulated receptor has thwarted investigations into such a receptor. Whereas the chemical and structural requirements which make a compound a good 3-MC-type inducer are strict and well-defined (Poland and Knutson, 1982; Safe *et al.*, 1985), the many different compounds that act as PB-type inducers exhibit great variety in chemical structure and have no obvious structural characteristics which would predict their efficacy or potency as enzyme inducers. PCN-type inducers, on the other hand, have been postulated to act via the glucocorticoid receptor to induce specific forms of P-450 (Schuetz and Guzelian, 1984).

4.2.3 Consequences of Induction

Many drugs act as autoinducers stimulating their own metabolism and thus reduce their own efficacy of which barbiturates are an example. At the same time, the metabolism of co-administered drugs may be altered (Park and Breckenridge, 1981). Generally, this is only of importance where the drugs have a low therapeutic index.

There may also be problems if induction of cytochrome P-450 species which generate toxic chemically-reactive intermediates occurs. Animal studies have shown that the hepatic necrosis produced by high doses of acetaminophen can be potentiated by PB and 3-MC (Mitchell *et al.*, 1984). Studies in mice (Nebert and Negishi, 1984) have shown that induction of cytochrome P450I can increase the conversion of pre-carcinogens, such as PAHs, to chemically reactive intermediates which may then covalently bind to cell components, producing toxicity or carcinogenicity.

4.3 PRELIMINARY PHARMACOKINETIC STUDIES

4.3.1 Introduction

In the present study, the pharmacokinetic parameters of three model compounds, antipyrine, theophylline and tolbutamide, were investigated in rats, when administered separately and concurrently. Antipyrine was chosen because it is frequently used to assess drug metabolizing enzyme activity and some selectivity has been observed in the induction of the pathways of antipyrine metabolism by different compounds (table 1.2). Theophylline and tolbutamide were chosen since the former has been postulated as a marker substrate for cytochromes P450I, inducible by PAHs, and the latter for cytochromes P450IIB, inducible by PB. Theophylline is known to show dose-dependent kinetics (Taunissen *et al.*, 1985a), therefore, its pharmacokinetics were studied at two different dose levels.

4.3.2 Experimental Design

Cannulated rats were administered a bolus dose, via the jugular vein, of antipyrine and/or theophylline in saline (2ml/Kg) or antipyrine and/or tolbutamide in PEG:PPG (9:1, 1ml/Kg). Blood samples were collected from the carotid artery (n=9, 250µl) over 180 min for antipyrine/theophylline and over 600 min for antipyrine/tolbutamide. Samples were assayed for antipyrine and/or theophylline (section 2.5.1, 2.5.2) or antipyrine and/or tolbutamide (section 2.5.3). A further study was conducted as above with tolbutamide alone in a group of β-NF induced rats (section 2.2.2.3).

4.3.3 Results and Discussion

4.3.3.1 *Antipyrine/Theophylline Pharmacokinetics*

The decline in theophylline concentration with time was mono-exponential with a half-life of about 150 min. No statistical difference (t-test)

Table 4.2 Pharmacokinetic Parameters of Antipyrine and Theophylline, Co-administered and Administered alone to Male Sprague-Dawley Rats

TREATMENT	CLEARANCE (ml/min/SRW)	VOLUME OF DISTRIBUTION (L/SRW)	HALF-LIFE (min)
Theophylline 3.25mg/Kg (n=4)	0.90 (0.25)	0.17 (0.03)	138.6 (39.8)
Theophylline 6.5 mg/Kg (n=4)	1.00 (0.27)	0.20 (0.03)	146.4 (27.5)
Theophylline 6.5 mg/Kg +	0.99 (0.22)	0.22 (0.04)	161.4 (27.5)
Antipyrine 50 mg/Kg (n=8)	1.88 (0.26)	0.23 (0.04)	84.4 (11.9)
Antipyrine 50 mg/Kg (n=8)	1.60 (0.33)	0.23 (0.02)	102.5 (29.9)

was observed when pharmacokinetic parameters such as clearance, half-life and apparent volume of distribution (table 4.2), generated by administering two different doses of theophylline (3.25 and 6.5mg/Kg), were compared. This indicated that at these doses, the pharmacokinetics of theophylline were linear.

The blood concentration-time curves for antipyrine and theophylline, when administered alone or when coadministered, were virtually superimposable (figures 4.1 and 4.2). This was also true for the dose normalized curves obtained from administering different doses of theophylline (3.25 and 6.5mg/Kg).

The pharmacokinetic data obtained after simultaneous administration of antipyrine and theophylline were not significantly different from those obtained after their separate administration (t-test) (Table 4.2). These data indicated that co-administration of antipyrine and

Figure 4.1 Antipyrine Blood Concentration-Time Curves following Administration of Antipyrine alone and with Theophylline

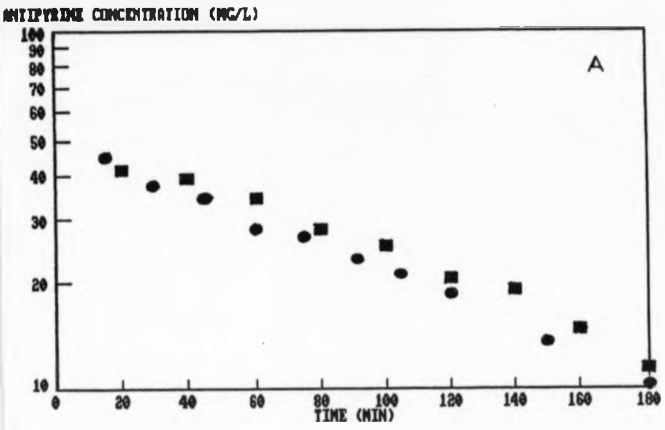
■ - antipyrine alone

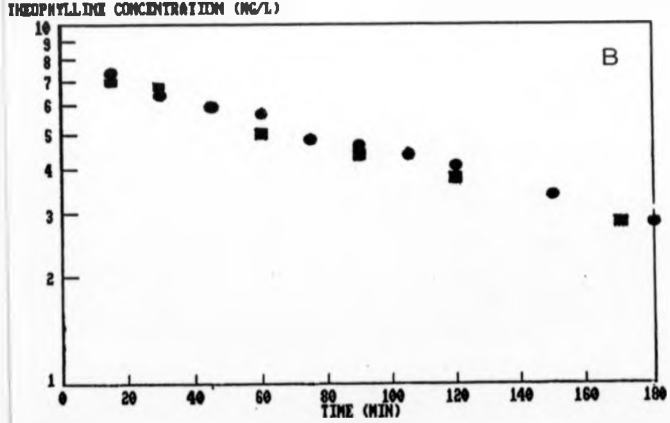
● - antipyrine with theophylline

Figure 4.2 Theophylline Blood Concentration-Time Curves following Administration of Theophylline alone and with Antipyrine

■ - theophylline alone

● - theophylline with antipyrine


ANTIPYRINE CONCENTRATION (MG/L)
A
TIME (MIN)


THEOPHYLLINE CONCENTRATION (MG/L)
B
TIME (MIN)

theophylline had no influence on their individual disposition kinetics.

4.3.3.2 *Antipyrine/Tolbutamide Pharmacokinetics*

Typical concentration-time curves generated from the antipyrine and tolbutamide experiments are shown in figures 4.3 and 4.4. Curves produced when the two drugs were simultaneously administered were identical to those obtained following separate administration of antipyrine and tolbutamide. Similarly, no significant difference (t-test) was observed in the pharmacokinetic parameters when tolbutamide or antipyrine were administered to rats alone or together (table 4.3).

Table 4.3 Pharmacokinetic Parameters of Tolbutamide and Antipyrine Coadministered and Administered alone in Control and β-NF-Induced Male Sprague-Dawley Rats

TREATMENT	CLEARANCE (ml/min/SRW)	VOLUME OF DISTRIBUTION (L/SRW)	HALF-LIFE (min)
Antipyrine 50 mg/Kg (n=6)	1.62 (32)	0.23 (0.02)	102.5 (29.9)
Antipyrine 50 mg/Kg + Tolbutamide 10 mg/Kg (n=5)	1.48 (21) 0.09 (0.02)	0.23 (0.02) 0.035 (0.002)	109.7 (13.6) 292.7 (64.9)
Tolbutamide 10 mg/Kg (n=6)	0.10 (0.02)	0.040 (0.008)	278.6 (46.1)
*Tolbutamide 10 mg/Kg (n=4)	0.12 (0.02)	0.052 (0.005)	292.6 (31.0)

* Rats induced with β-NF 100mg/Kg ip x 3 daily doses.

Figure 4.3 Antipyrine Plasma Concentration-Time Curves following Administration of Antipyrine alone and with Tolbutamide

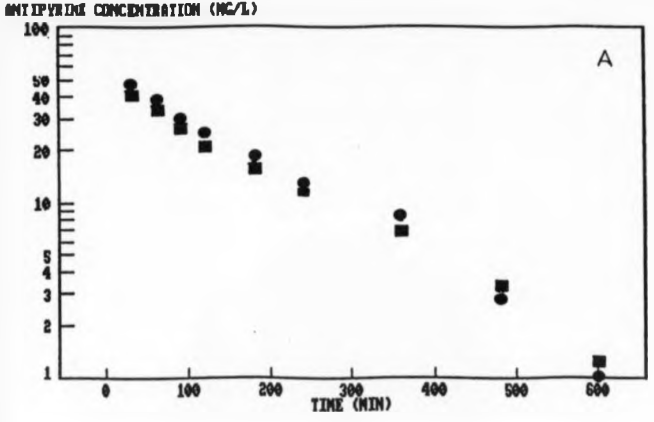
■ - antipyrine alone

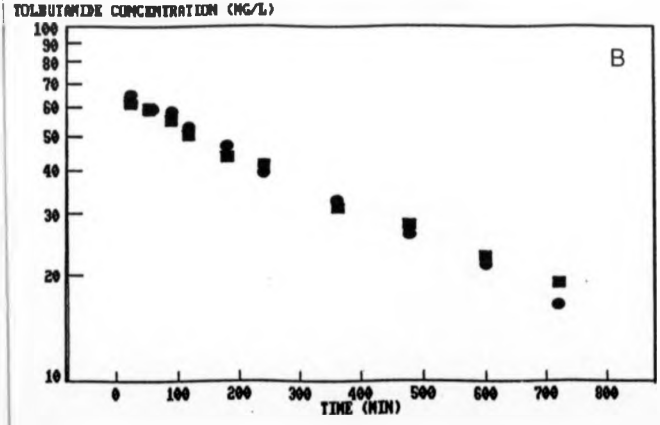
● - antipyrine with tolbutamide

Figure 4.4 Tolbutamide Plasma Concentration-Time Curves following Administration of Tolbutamide alone and with Antipyrine

■ - tolbutamide alone

● - tolbutamide with antipyrine


ANTIPYRINE CONCENTRATION (MG/L)
100
50
40
30
20
10
5
4
3
2
1
A
0
100
200
300
400
500
600
TIME (MIN)


TOLBUTAMIDE CONCENTRATION (MG/L)
100
90
80
70
60
50
40
30
20
10
B
0
100
200
300
400
500
600
700
800
TIME (MIN)

Although β-NF pretreatment produced slight increases in the clearance, half-life and apparent volume of distribution of tolbutamide in rats, these were not significant (Student's t test) (table 4.3).

4.3.4 Conclusions

The drug metabolizing ability of an individual is sensitive to a large number of variables. Day to day variability in such activity can confound comparison of the disposition of different drugs at different times (Rowland, 1980). It has been suggested that by co-administering investigative drugs, time-dependant variables such as liver blood flow and cytochrome P-450 activity may be avoided (Breimer, 1983; Van der Graaff *et al.*, 1983a).

The potential for competition between two extensively metabolized, simultaneously administered drugs for the isoenzymes of cytochrome P-450 exists, but use of low doses of such compounds may circumvent this problem (Breimer *et al.*, 1984; Van der Graaff *et al.*, 1983a). The aim of these preliminary studies was to identify whether, at the doses of antipyrine, theophylline and tolbutamide chosen, such an interaction was detectable in male Sprague-Dawley rats. This was particularly important since theophylline has been shown to exhibit capacity limited kinetics (Taunissen *et al.*, 1985a) and tolbutamide is highly bound to plasma proteins (Judis, 1972).

When Taunissen *et al.* (1985a) studied the dose-dependency of theophylline kinetics in male Wistar rats, linear kinetics were observed for doses below 11 mg/Kg. Since the metabolism of antipyrine is slower in male Sprague-Dawley rats than in male Wistar rats it was likely that theophylline metabolism would also be different. Indeed, theophylline half-life was found to be 150min (male Sprague-Dawley rats) as opposed to 70min (male Wistar rats). To ensure that the dose chosen for further studies was in the linear region and also would produce measurable

levels following induction of drug metabolism, two theophylline doses were assessed in rats. Linear pharmacokinetics were observed for both doses (3.25 and 6.5mg/Kg). The higher dose was therefore chosen for further studies.

These preliminary studies indicated that co-administration of theophylline (6.5mg/Kg) or tolbutamide (10mg/Kg) with antipyrine (50mg/Kg) to rats did not affect their individual pharmacokinetics. Further studies were therefore carried out using these combinations. Although these data have been confirmed by another study in the rat (Teunissen *et al.*, 1986) and in human voluntaers (Teunissen *et al.*, 1985b) a subsequent study in humans has suggested that the co-administration of antipyrine (18mg/Kg) and theophylline (5mg/Kg) reduces the clearance of both compounds without affecting their apparent volumes of distribution (Denlinger *et al.*, 1987). These conflicting reports indicate the importance of such preliminary investigations. The use of tolbutamide as a probe for measuring drug metabolizing ability has not been as extensive as for the other two compounds. Although it was known that PB induced the metabolism of tolbutamide (Back *et al.*, 1984), the effect of β-NF induction was not known. No significant difference was observed between the pharmacokinetic parameters obtained for tolbutamide in untreated and β-NF pretreated rats. This indicated that β-NF-inducible isoenzymes of cytochrome P-450 were not involved in the metabolism of tolbutamide. This supported the idea that tolbutamide might act as a suitable *in-vivo* marker for PB-inducible forms of cytochrome P-450.

4.4 EFFECT OF β-NF ADMINISTRATION ON *IN-VIVO* AND *IN-VITRO* MARKERS OF DRUG METABOLIZING CAPACITY MEASURED IN RATS

4.4.1 Introduction

The aim of this study was to produce a wide range of drug metabolizing enzyme activities in rats using the inducing agent β-NF whilst keeping extrinsic factors constant. These animals were then used to investigate the sensitivity and selectivity of a number of *in-vivo* model compounds and *in-vitro* substrates as markers of drug metabolizing enzyme activity. Each animal was used for both the *in-vivo* and the *in-vitro* studies in order to eliminate errors through inter-individual differences in response to different doses of the inducing agent.

4.4.2 Experimental Design

Male Sprague-Dawley rats (n=16, 230-280g) were administered various daily ip doses of β-NF (0-100mg/Kg; 9.00-10.00 h) in corn oil (2ml/Kg) for three consecutive days. Rats were cannulated (section 2.4.2) after the third dose (section 2.4.2). On the fourth day they were placed in restraining cages. A solution of (N-methyl-^{14}C)-antipyrine (50mg/Kg, 1μCi/Kg) and theophylline (6.5mg/Kg) in normal saline (2ml/Kg) was administered as a bolus over 1 min, *via* the jugular vein. Mannitol (7.5g/100ml in normal saline 0.9% w/v) was infused (1.5ml/h) into the jugular vein throughout the experiment, in order to ensure a regular flow of urine.

Blood samples (n=9, 250μl) were collected over 3 h and urine over 4 h. Following completion of sampling, the rats were immediately killed by cervical dislocation, their bladders drained and liver microsomes prepared (section 2.3.1). Blood samples were assayed for antipyrine and theophylline (sections 2.5.1 and 2.5.2) and urine samples for antipyrine and its metabolites (section 2.5.5). Data was analysed as described in section 2.6.

The following clearances were determined - antipyrine total clearance CL(AP), theophylline total clearance CL(TH), and 4-hydroxyantipyrine CL(4H), norantipyrine CL(N) and 3-hydroxymethyl-antipyrine formation clearances CL(3H) (equation 1.9).

In-vitro parameters were determined, in prepared liver microsomes, as follows; microsomal protein (section 2.3.2), cytochrome P-450 content (section 2.3.3), ECOD activity (section 2.3.4), MCOD activity (section 2.3.5), EROD activity (section 2.3.6) and ALE activity (section 2.3.7).

4.4.3 Results and Discussion

Typical concentration-time curves for antipyrine and theophylline in control and maximally induced rats are shown in figures 4.5 and 4.6. They were adequately described by a monoexponential model. The marked decrease in half-life confirmed the sensitivity of both compounds to β-NF induction. No alteration in apparent volume of distribution was observed for either antipyrine or theophylline (figure 4.7) following β-NF induction.

Table 4.4 summarises the effects of β-NF induction on *the in-vivo* and *in-vitro* parameters. Following induction with β-NF, large changes were noted in CL(TH) (10 fold), CL(AP) (4 fold), CL(4H) (6 fold) and CL(N) (5 fold). No trends were observed with CL(3H). For the *in-vitro* markers large changes were noted for ECOD (14 fold), EROD (103 fold) and smaller increases in P-450 (3 fold) were observed. MCOD showed little response whilst ALE showed a negative response (50% of control).

4.4.3.1 *β-NF Dose Response Curves*

Figures 4.8 and 4.9 show the typical responses of the parameters measured, to the β-NF dose administered. Whilst CL(3H) values were unchanged following β-NF induction, a hyperbolic function described the β-NF dose-responses observed for the other *in-vivo* parameters, CL(TH),

Figure 4.5 Antipyrine Blood Concentration-Time Curves in Control and β-NF Induced Rats (100mg/Kg)

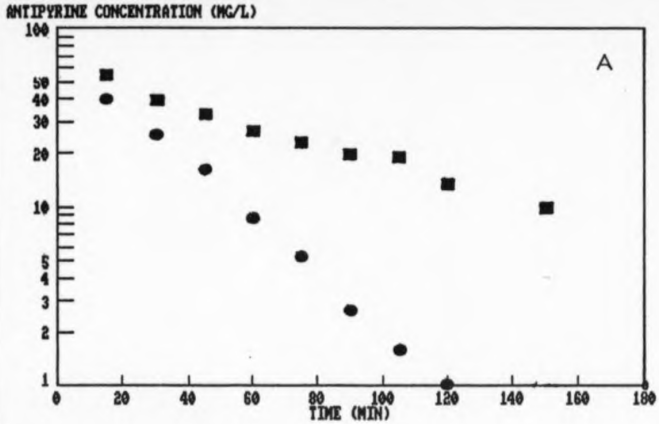
■ - Control

● - β-NF induced

Figure 4.6 Theophylline Blood Concentration-Time Curves in Control and β-NF Induced Rats (100mg/Kg)

■ - Control

● - β-NF induced


ANTIPYRINE CONCENTRATION (MG/L)
100
50
40
30
20
10
5
4
3
2
1
A
0
20
40
60
80
100
120
140
160
180
TIME (MIN)

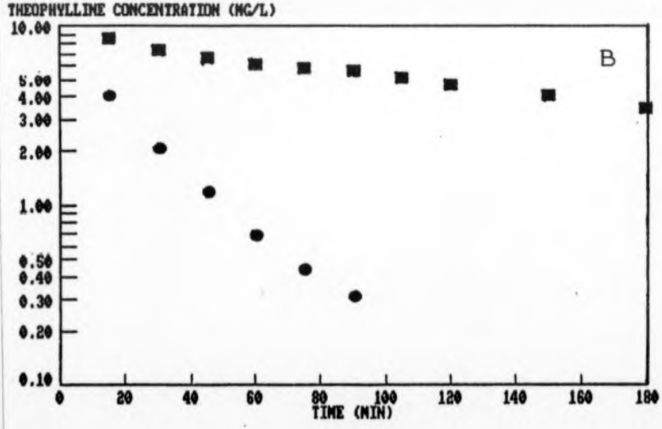
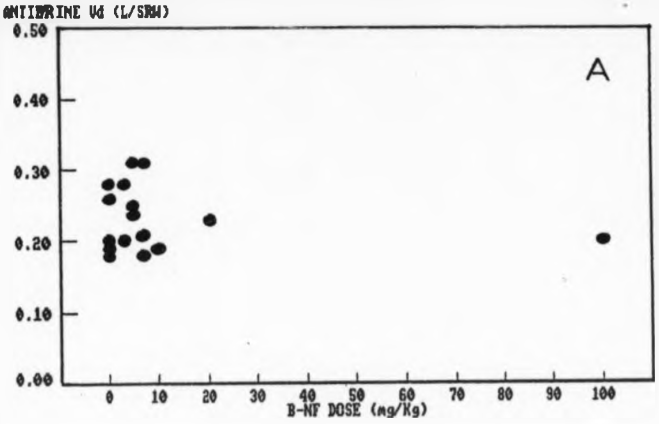

THEOPHYLLINE CONCENTRATION (MG/L)
10.00
5.00
4.00
3.00
2.00
1.00
0.50
0.40
0.30
0.20
0.10
B
0
20
40
60
80
100
120
140
160
180
TIME (MIN)

Figure 4.7 Effect of β-NF Induction on the Apparent Volumes of Distribution of Antipyrine and Theophylline

A - Apparent V_d of Antipyrine versus β-NF Dose

B - Apparent V_d of Theophylline versus β-NF Dose


ANTIPYRINE Vd (L/SKG)
0.50
0.40
0.30
0.20
0.10
0.00
A
0
10
20
30
40
50
60
70
80
90
100
B-NF DOSE (mg/Kg)

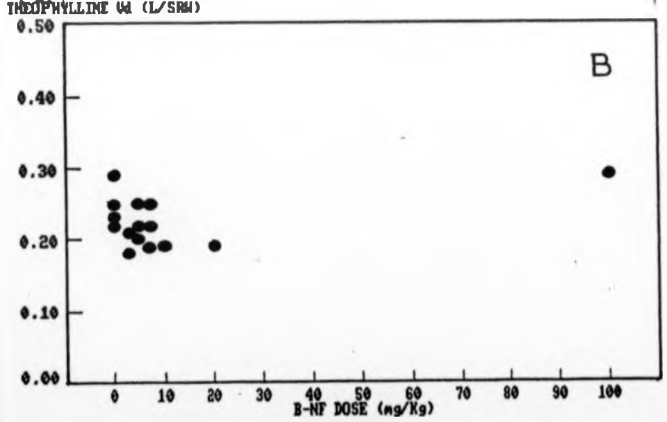

THEOPHYLLINE Vd (L/SKG)
0.50
0.40
0.30
0.20
0.10
0.00
B
0
10
20
30
40
50
60
70
80
90
100
B-NF DOSE (mg/Kg)

Table 4.4 Effect of β-NF Induction on Various Markers of Drug Metabolizing Capacity

PARAMETER	n	CONTROL	β-NF 100mg/kg INDUCTION	FOLD INCREASE
*CL(TH)	16	1.11 (0.17)	10.63	9.6
*CL(AP)	16	1.87 (0.32)	6.51	3.5
*CL(4H)	15	0.23 (0.11)	1.45	6.3
*CL(H)	15	0.15 (0.06)	0.81	5.4
*CL(3H)	15	0.27 (0.08)	0.22	0.8
#ECOD	16	0.320 (0.049)	4.451	14
#EROD	16	0.056 (0.015)	5.748	103
^P-450	16	0.374 (0.023)	0.922	2.5
#MCOD	16	0.192 (0.018)	0.242	1.3
#ALE	16	1.770 (0.252)	0.651	0.4

n number of rats included in study
* ml/min/SKW
# nmole/min/mg microsomal protein
^ nmole/mg microsomal protein

CL(AP), CL(H) and CL(4H). CL(AP), CL(H) and CL(4H) values rose sharply with β-NF dose administered, reaching a maximum at about 20mg/Kg whereas CL(TH) values increased gradually up to the maximum β-NF dose administered.

No trend was observed between MCOD activity and β-NF dose administered. With the exception of ALE activity which decreased with β-NF dose and reached a minimum with doses of about 20mg/Kg, the *in-vitro* parameters, ECOD, EROD and P-450 increased with dose and peaked at about 20mg/Kg. In order to achieve a wide range in activities, β-NF doses were concentrated in the range 0 to 20mg/Kg.

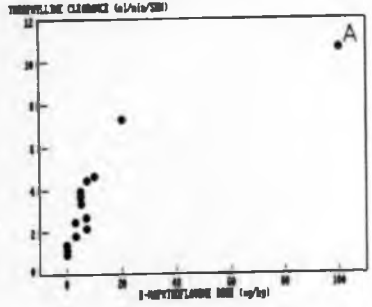
Figure 4.8 The Response of *In-vivo* Markers of Drug Metabolizing Capacity to Induction with various Doses of β-NF

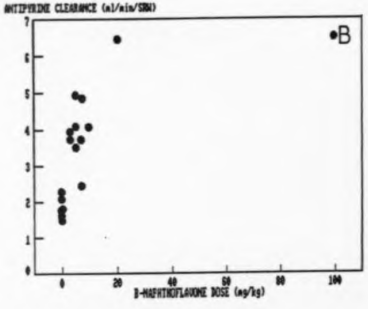
A - Theophylline Clearance versus β-NF Dose

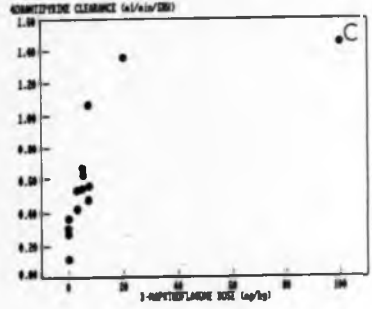
B - Antipyrine Clearance versus β-NF Dose

C - 4-OHAntipyrine Clearance versus β-NF Dose

D - Norantipyrine Clearance versus β-NF Dose


A
3-HAPTHOFLAVONE DOSE (mg/kg)


B
3-HAPTHOFLAVONE DOSE (mg/kg)


C
3-HAPTHOFLAVONE DOSE (mg/kg)

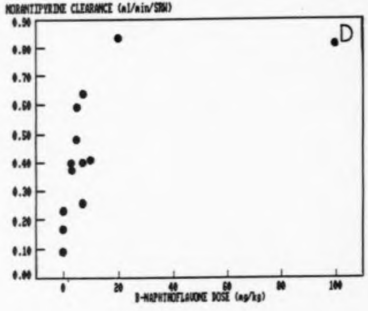

D
3-HAPTHOFLAVONE DOSE (mg/kg)

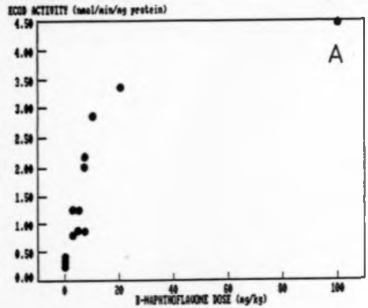
Figure 4.9 The Response of *In-vitro* Markers of Drug Metabolizing Capacity to Induction with various Doses of β-NF

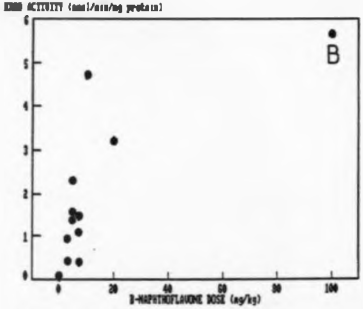
A - ECOD Activity versus β-NF Dose

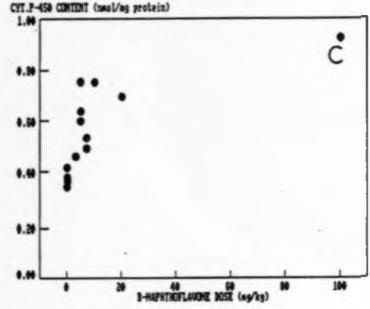
B - EROD Activity versus β-NF Dose

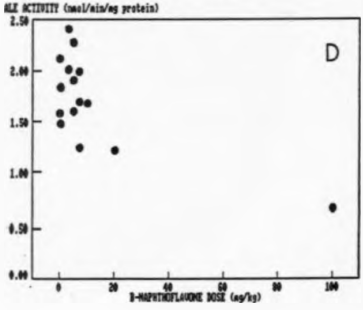
C - P-450 Content versus β-NF Dose

D - ALE Activity versus β-NF Dose


ECOD ACTIVITY (nmol/min/mg protein)
A
B-NAPHTHOFLAVONE DOSE (mg/kg)


MROD ACTIVITY (nmol/min/mg protein)
B
B-NAPHTHOFLAVONE DOSE (mg/kg)


CYT.P-450 CONTENT (nmol/mg protein)
C
B-NAPHTHOFLAVONE DOSE (mg/kg)


ALK ACTIVITY (nmol/min/mg protein)
D
B-NAPHTHOFLAVONE DOSE (mg/kg)

4.4.3.2 *Correlations between In-vivo and In-vitro Parameters*

Table 4.5 illustrates correlations observed between and within the various *in-vivo* and *in-vitro* markers measured. Examples of correlations are shown in figures 4.10, 4.11, 4.12 and 4.13.

4.4.3.2.1 *Correlations between In-vivo Parameters*

With the exception of CL(3H) (r-range, 0.13-0.41), a marked level of covariance was demonstrated between all of the *in-vivo* parameters (r-range, 0.88-0.99). Apart from the CL(3H) relationships, the lowest correlation was observed between CL(AP) and CL(TH) (r=0.88). CL(AP) correlated more strongly with CL(4H) (r=0.94), however CL(M) seemed to predict CL(AP) better than any of the other *in-vivo* parameters (r=0.99).

These data, together with the fact that there appeared to be more similarity between CL(4H) and CL(TH) (r=0.94) than between any of the other *in-vivo* parameters and CL(TH), indicated that there are differences between the P-450 isoenzymes involved in the different metabolic pathways of antipyrine (figure 4.10).

The lack of correlation between CL(3H) and other *in-vivo* parameters is consistent with the selective induction pattern of β-NF previously documented using antipyrine metabolite kinetics (table 1.2).

4.4.3.2.2 *Correlations between In-vitro Parameters*

Since MCOD activity was unaffected by β-NF induction, it was not surprising that correlations between this and the other *in-vitro* parameters were poor (r-range, 0.29 - 0.55). Unlike the other parameters, ALE activity decreased with increasing β-NF dose administered, thus negative but poor correlations were also observed between this and the others parameters (r-range, 0.43 - 0.65). Examples of relationships between ECOD and MCOD/ALE activities are shown in figure 4.11.

The other three parameters, P-450 content, and ECOD and EROD activities were more highly correlated. Of these the poorest relationship was observed between ECOD activity and P-450 content (r=0.80) and the highest between ECOD and EROD activities (r=0.92) (figure 4.11).

The high correlation between P-450 and EROD activity (r=0.90) was surprising, since, although specific forms of P-450 may be induced over 100 fold, P-450 generally only increases 3-fold with maximal induction. P-450 content in control animals also reflects the amount of constitutive cytochromes P-450 present and it is known that levels of PAH-inducible cytochromes P450I are very low in the non-induced animal, as reflected in the low EROD activity in untreated animals.

4.4.3.2.3 *Correlations between In-vivo and In-vitro Parameters*

The poor correlations observed between CL(3H) and the *in-vitro* parameters, and MCOD and ALE activities and the *in-vivo* parameters were to be expected from the preceding data. Clearly these parameters were not predictive for changes in drug metabolizing activity resulting from β-NF induction.

Of the *in-vivo* parameters, CL(TH) stood out as correlating most highly with P-450 content (r=0.86), EROD (r=0.91) and ECOD activities (r=0.95). Next to this, CL(4H) appeared also to correlate highly with the *in-vitro* parameters, EROD (r=0.89) and ECOD activities (r=0.93). These also followed the trends described previously. Clearly the parameters CL(TH) and CL(4H) and ECOD and EROD activities can act as sensitive probes for β-NF induction (figures 4.12 and 4.13).

Although CL(H) produced slightly better correlations with the *in-vitro* parameters than CL(AP), not unexpectedly, these two parameters were much poorer as indicators of β-NF induction than CL(TH) or CL(4H).

Table 4.5 Relationships Between and Within *In-vivo* and *In-vitro Markers* of Drug Metabolizing Capacity Measured in Control and β-NF induced Rats

CL(AP)	CL(4H)	CL(H)	CL(3H)	ALE	MCOD	P-450	ECOD	EROD	
0.884	0.944	0.890	0.129	-0.613	0.350	0.866	0.951	0.910	CL(TH)
	0.941	0.987	0.395	-0.310	0.622	0.781	0.808	0.764	CL(AP)
		0.956	0.372	-0.551	0.399	0.783	0.930	0.890	CL(4H)
			0.409	-0.348	0.621	0.826	0.827	0.845	CL(H)
				0.269	0.418	0.019	0.149	0.040	CL(3H)
					0.332	-0.426	-0.648	-0.539	ALE
						0.546	0.287	0.441	MCOD
							0.796	0.897	P-450
								0.915	ECOD

The values represented in this table are correlation coefficients.

Critical values of r $p < 0.001$; n = 16, 0.725; n = 15, 0.704
n=16 for CL(TH), CL(AP) and the *in-vitro* parameters
n=15 for the formation clearance data

The following show correlation coefficients and their corresponding coefficients of determination (%)

$r = 0.95$, $r^2 = 90\%$
$r = 0.90$, $r^2 = 80\%$
$r = 0.84$, $r^2 = 70\%$
$r = 0.77$, $r^2 = 60\%$

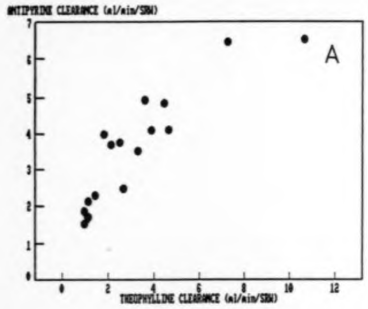
Figure 4.10 Correlations between Theophylline Clearance, and other *In-vivo* Markers of Drug Metabolizing Capacity in Control and β-NF Induced Rats

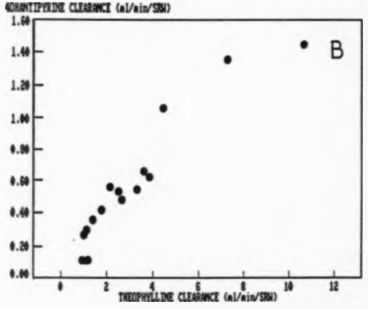
A - Antipyrine Clearance versus Theophylline Clearance

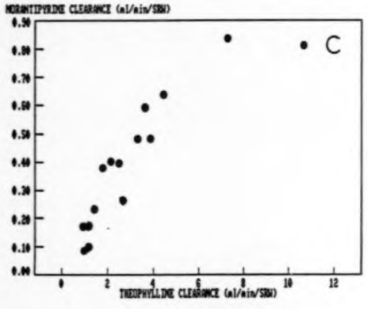
B - 4-OHAntipyrine Formation Clearance versus Theophylline Clearance

C - Norantipyrine Formation Clearance versus Theophylline Clearance

D - 3-OHMeAntipyrine Formation Clearance versus Theophylline Clearance


ANTIPYRINE CLEARANCE (ml/min/SRA)
A
THEOPHYLLINE CLEARANCE (ml/min/SRA)


4OHANTIPYRINE CLEARANCE (ml/min/SRA)
B
THEOPHYLLINE CLEARANCE (ml/min/SRA)


NORANTIPYRINE CLEARANCE (ml/min/SRA)
C
THEOPHYLLINE CLEARANCE (ml/min/SRA)

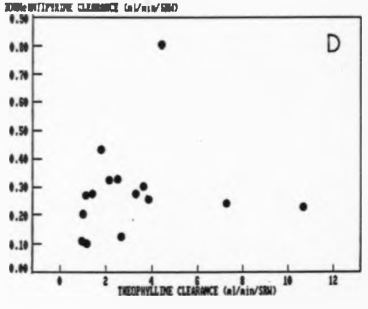

3OHNorANTIPYRINE CLEARANCE (ml/min/SRA)
D
THEOPHYLLINE CLEARANCE (ml/min/SRA)

Figure 4.11 Correlations between ECOD Activity and other *In-vitro* Markers of Drug Metabolizing Capacity in Control and β-NF Induced Rats

A - EROD Activity versus ECOD Activity

B - P-450 Content versus ECOD Activity

C - MCOD Activity versus ECOD Activity

D - ALE Activity versus ECOD Activity

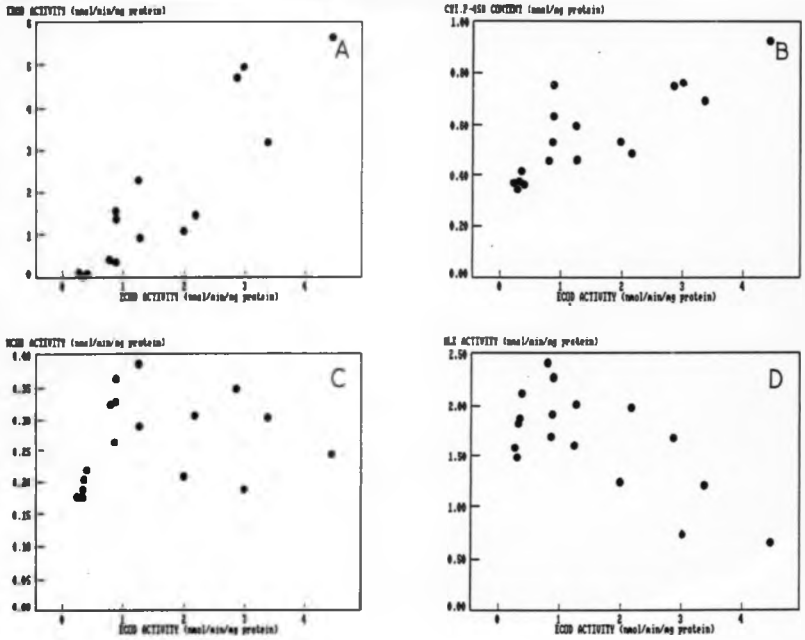

ECOD ACTIVITY (nmol/min/mg protein)
A
ECOD ACTIVITY (nmol/min/mg protein)
CYT.P-450 CONTENT (nmol/mg protein)
B
ECOD ACTIVITY (nmol/min/mg protein)
NCOD ACTIVITY (nmol/min/mg protein)
C
ECOD ACTIVITY (nmol/min/mg protein)
NLE ACTIVITY (nmol/min/mg protein)
D
ECOD ACTIVITY (nmol/min/mg protein)

Figure 4.12 Correlations between Theophylline Clearance and various *in-vitro* Markers of Drug Metabolizing Capacity in Control and β-NF Induced Rats

A - Theophylline Clearance versus ECOD Activity

B - Theophylline Clearance versus EROD Activity

C - Theophylline Clearance versus P-450 Content

D - Theophylline Clearance versus ALE Activity

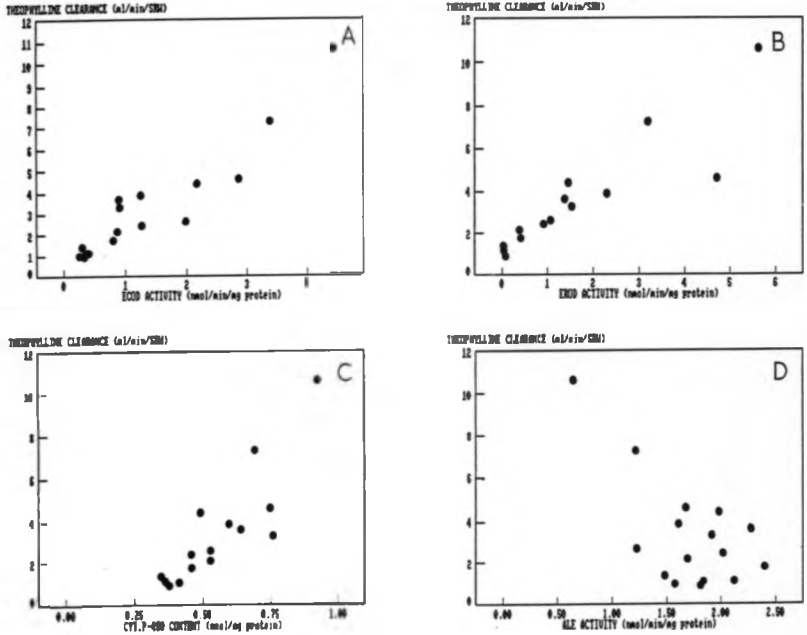

THEOPHYLLINE CLEARANCE (ml/min/SKM)
A
ECOD ACTIVITY (nmol/min/mg protein)
THEOPHYLLINE CLEARANCE (ml/min/SKM)
B
ENOD ACTIVITY (nmol/min/mg protein)
THEOPHYLLINE CLEARANCE (ml/min/SKM)
C
CYT.P-450 CONTENT (nmol/mg protein)
THEOPHYLLINE CLEARANCE (ml/min/SKM)
D
ALE ACTIVITY (nmol/min/mg protein)

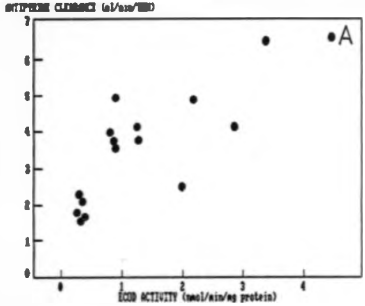
Figure 4.13 Correlations between ECOD Activity and various *In-vivo* Markers of Drug Metabolizing Capacity in Control and β-NF Induced Rats

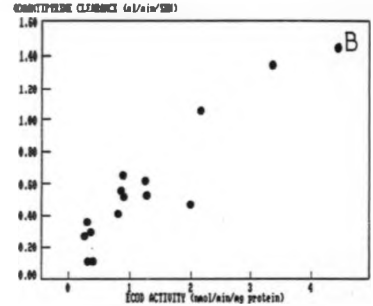
A - Antipyrine Clearance versus ECOD Activity

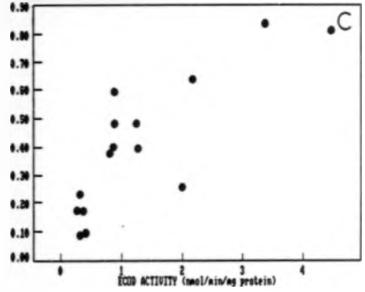
B - 4OHAntipyrine Formation Clearance versus ECOD Activity

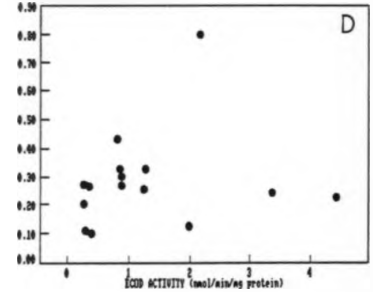
C - Norantipyrine Formation Clearance versus ECOD Activity

D - 3OHMeAntipyrine Formation Clearance versus ECOD Activity


A
ECOD ACTIVITY (nmol/min/mg protein)


B
ECOD ACTIVITY (nmol/min/mg protein)


C
ECOD ACTIVITY (nmol/min/mg protein)


D
ECOD ACTIVITY (nmol/min/mg protein)

4.5 EFFECT OF PB ADMINISTRATION ON *IN-VIVO* AND *IN-VITRO* MARKERS OF DRUG METABOLIZING CAPACITY IN THE RAT

4.5.1 Introduction

In the previous study, a number of *in-vivo* and *in-vitro* markers were investigated with respect to their sensitivity and selectivity as markers of drug metabolizing ability in a group of β-NF induced rats. As expected, the markers which previous studies have indicated to be specific for cytochromes P450I, CL(TH) and EROD activity, did indeed correlate highly. To test further the hypothesis that the use of markers specific for certain inducible forms of cytochrome P-450 improves correlations observed, a similar study was carried out in a group of PB-inducible rats.

Since CL(TH) was found to be a good marker for cytochromes P-450I, its value as a marker for cytochromes P450IIB was open to question. Therefore a further study was conducted but using another marker, tolbutamide, thought to be specific for PB induction.

4.5.2 Experimental Design

Sprague-Dawley rats (15-study 1 and 16-study 2, 230-280g) were given various daily ip doses of PB (0-100mg/Kg, in normal saline (2ml/Kg) for three consecutive days. Rats were cannulated (section 2.4.2) after the third dose. On the fourth day, they were placed in restraining cages and received one of the following treatments;

Study 1:- (N-methyl-^{14}C)-antipyrine (50mg/Kg, 1μCi/Kg) and theophylline (6.5mg/Kg) in normal saline (2ml/Kg) *via* the jugular vein. Blood samples (n=9, 250μl) were collected over 3 h and urine over 4 h. Mannitol (7.5g/100ml) in normal saline was infused (1.5ml/h) into the jugular vein throughout the experiment, in order to ensure a regular flow of urine.

Study 2:- antipyrine (50mg/Kg) and tolbutamide (10mg/Kg) in polyethylene glycol : polypropylene glycol (9:1, 1ml/kg) *via* the jugular vein. Blood samples (n=9, 250µl) were collected over 6 h.

Rats were then removed from their restraining cages and killed by cervical dislocation. Bladders from rats in the 1st study were drained before preparation of liver microsomes (section 2.3.1).
Blood samples from the first study were assayed for theophylline and antipyrine (section 2.5.1 and 2.5.2) and urine samples for antipyrine metabolites (section 2.5.5). Blood samples from the second study were assayed for tolbutamide and antipyrine (section 2.5.3). Data were analysed as described in section 2.6.

The following clearances were calculated;
Study 1:- antipyrine total clearance CL(AP), theophylline total clearance CL(TH), 4-hydroxyantipyrine CL(4H), norantipyrine CL(N) and 3hydroxymethylantipyrine CL(3H) formation clearances..
Study 2:- antipyrine total clearance CL(AP) and tolbutamide CL(TOL)

The following *in-vitro* assays were carried out, microsomal protein (section 2.3.1), cytochrome P-450 content (section 2.3.3), ECOD activity (section 2.3.4), NCOD activity (section 2.3.5), ALK activity (section 2.3.7) and in study 1, EROD activity (section 2.3.6).

4.5.3 Results and Discussion

Typical profiles for antipyrine, theophylline and tolbutamide in control and maximally induced rats are shown in figures 4.14, 4.15, 4.16 and 4.17. The kinetics of these three compounds in control and induced rats

Figure 4.14 Antipyrine Blood Concentration-Time Curves in Control and PB Induced Rats

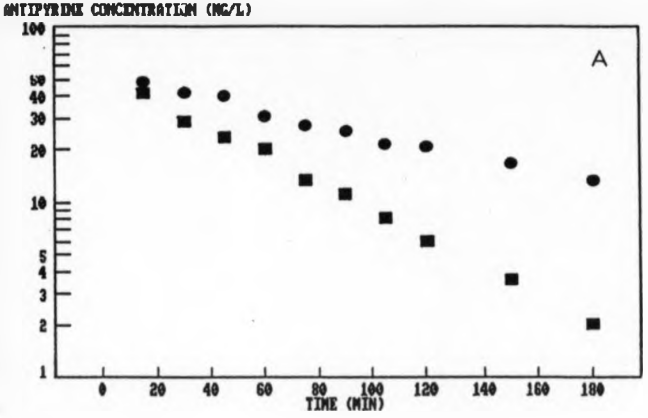
● - control

■ - PB induced

Figure 4.15 Theophylline Blood Concentration-Time Curves in Control and PB Induced Rats

● - control

■ - PB induced


ANTIPYRINE CONCENTRATION (MG/L)
100
50
40
30
20
10
5
4
3
2
1
A
0
20
40
60
80
100
120
140
160
180
TIME (MIN)

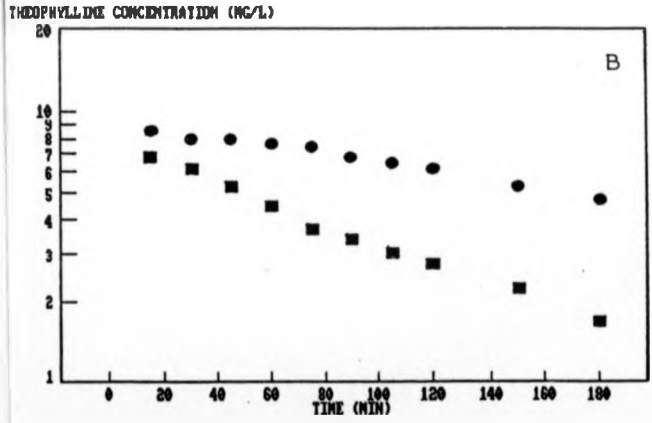

THEOPHYLLINE CONCENTRATION (MG/L)
20
10
9
8
7
6
5
4
3
2
1
B
0
20
40
60
80
100
120
140
160
180
TIME (MIN)

Figure 4.16 Antipyrine Plasma Concentration-Time Curves in Control ♂ PB Induced Rats (80 mg/kg)

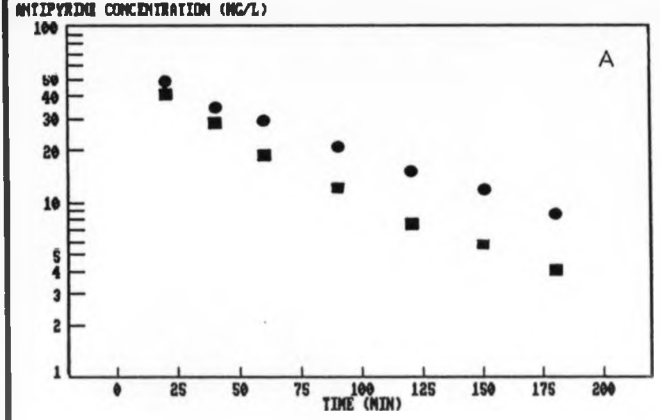
● - control

■ - PB induced

Figure 4.17 Tolbutamide Plasma Concentration-Time Curves in Control ♂ PB Induced Rats (80 mg/kg)

● - control

■ - PB induced


ANTIPYRINE CONCENTRATION (MG/L)
100
50
40
30
20
10
5
4
3
2
1
A
0
25
50
75
100
125
150
175
200
TIME (MIN)

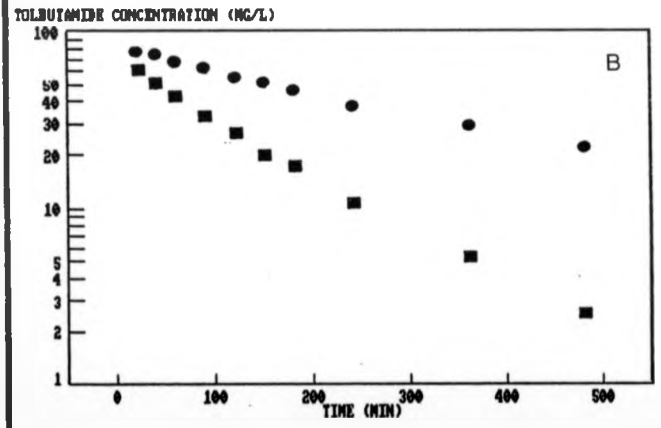

TOLBUTAMIDE CONCENTRATION (MG/L)
100
50
40
30
20
10
5
4
3
2
1
B
0
100
200
300
400
500
TIME (MIN)

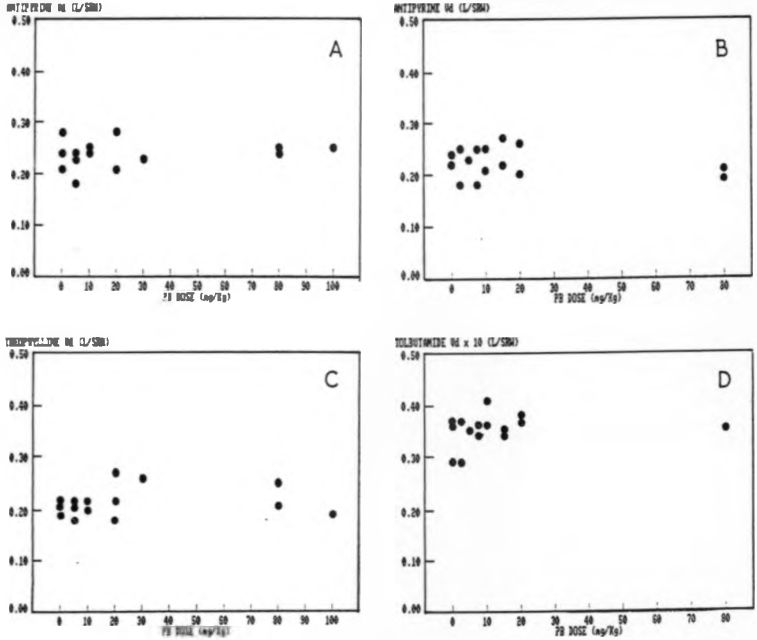
Figure 4.18 Effect of PB Induction on the Apparent Volumes of Distribution of Antipyrine Theophylline and Tolbutamide

Study 1 A - Apparent V_d of Antipyrine versus PB Dose

C - Apparent V_d of Theophylline versus PB Dose

Study 2 B - Apparent V_d of Antipyrine versus PB Dose

D - Apparent V_d of Tolbutamide versus PB Dose


ANTIPYRINE Cl (L/SRM)
A
0.50
0.40
0.30
0.20
0.10
0.00
0 10 20 30 40 50 60 70 80 90 100
PB DOSE (mg/Kg)
ANTIPYRINE Cl (L/SRM)
B
0.50
0.40
0.30
0.20
0.10
0.00
0 10 20 30 40 50 60 70 80
PB DOSE (mg/Kg)
C
0.50
0.40
0.30
0.20
0.10
0.00
0 10 20 30 40 50 60 70 80 90 100
TOLBUTAMIDE Cl x 10 (L/SRM)
D
0.50
0.40
0.30
0.20
0.10
0.00
0 10 20 30 40 50 60 70 80
PB DOSE (mg/Kg)

was adequately described as a monoexponential decline. The decrease observed in the half-lives of antipyrine and theophylline was less dramatic following maximal induction with β-NF than with PB. However tolbutamide elimination was only increased following PB induction. No alteration in the apparent volume of distribution of any of the three compounds was observed following PB-induction (figure 4.18).

Tables 4.6 and 4.7 summarise the effects of PB induction on the *in-vivo* and *in-vitro* parameters measured in studies 1 and 2 respectively. The parameters CL(N), CL(3H) and EROD activity showed a mean increase which was not much greater than the variability observed within control animals. Significant increases were observed in all of the parameters following PB induction (2 - 5 fold). The largest changes occurred in ECOD (4.5 - 5 fold) and MCOD activity (4 - 5 fold).

4.5.3.1 *PB Dose response curves*

Figures 4.19 and 4.20 show typical responses of the parameters measured to the dose of PB administered to the rats. Data from the two PB induction studies were combined. With the exception of CL(3H), CL(N) and EROD activity, the PB dose response curves produced by the parameters measured were hyperbolic in shape. A striking difference was seen between the PB and β-NF dose responses. The increase in the parameters measured was much more rapid and the peak was reached earlier with β-NF (20mg/Kg) than with PB (30mg/Kg). There was also more scatter in the PB data.

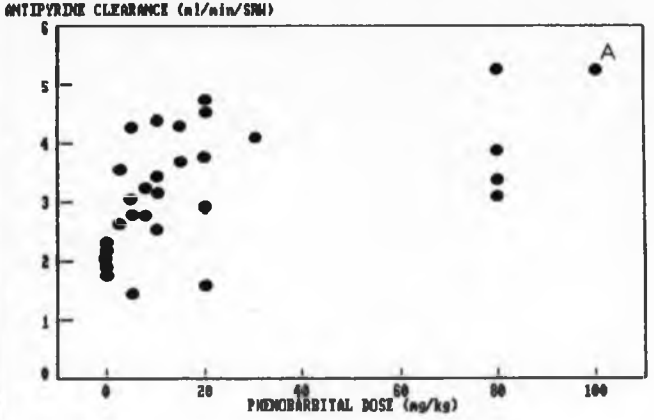
4.5.3.2 *Correlations between In-vivo and In-vitro Parameters*

Tables 4.6 and 4.7 illustrate correlations obtained between and within the various in-vivo and in-vitro markers. Examples of correlations are shown in figures 4.21 to 4.27.

Figure 4.19 The Response of *In-vivo* Markers of Drug Metabolizing Capacity to Induction by various Doses of PB

A - Antipyrine Clearance versus PB Dose

B - Tolbutamide Clearance versus PB Dose


ANTIPYRINE CLEARANCE (ml/min/SRW)
A
PHENOBARBITAL DOSE (mg/kg)

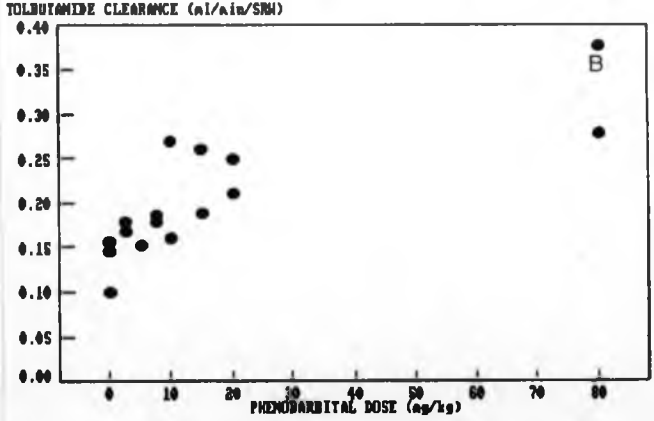

TOLBUTAMIDE CLEARANCE (ml/min/SRW)
B
PHENOBARBITAL DOSE (mg/kg)

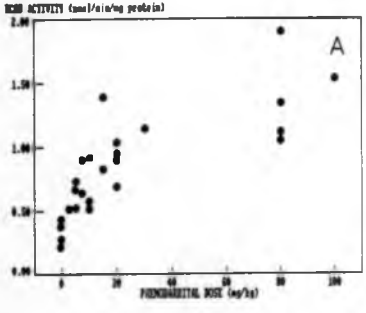
Figure 4.20 The Response of *In-vitro* Markers of Drug Metabolizing Capacity to Induction by various Doses of PB

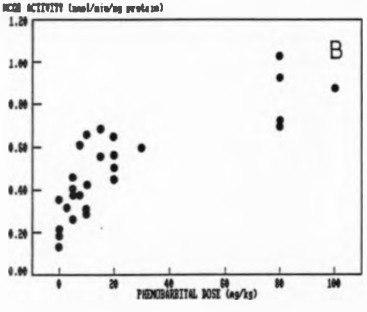
A - ECOD Activity versus PB Dose

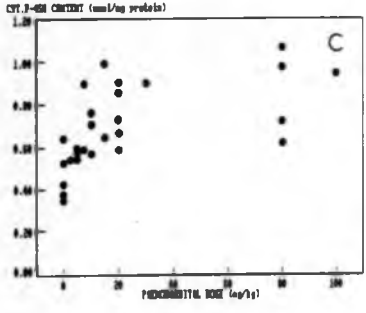
B - MCOD Activity versus PB Dose

C - P-450 Content versus PB Dose

D - ALE Activity versus PB Dose


HCDH ACTIVITY (nmol/min/mg protein)
A
PHENOBARBITAL DOSE (mg/kg)


HCDH ACTIVITY (nmol/min/mg protein)
B
PHENOBARBITAL DOSE (mg/kg)


CYT.P-450 CONTENT (nmol/mg protein)
C
PHENOBARBITAL DOSE (mg/kg)

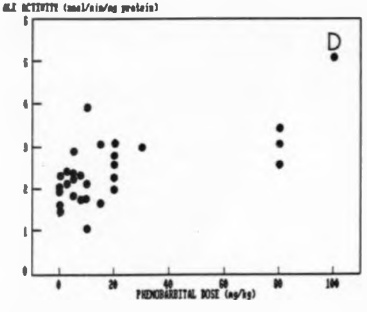

ALA ACTIVITY (nmol/min/mg protein)
D
PHENOBARBITAL DOSE (mg/kg)

Table 4.6 Effect of PB Induction on various Markers of Drug Metabolizing Capacity (Antipyrine-Theophylline Study)

PARAMETER	n	CONTROL	PB 80mg/Kg INDUCTION	FOLD INCREASE
[a]CL(TH)	15	0.83 (0.06)	2.10	2.5
[a]CL(AP)	15	1.92 (0.16)	5.25	2.7
[a]CL(4H)	15	0.23 (0.06)	0.62	2.7
[a]CL(N)	15	0.17 (0.04)	0.27	1.6
[a]CL(3H)	15	0.22 (0.05)	0.31	1.4
[b]ECOD	15	0.293 (0.088)	1.530	5.2
[b]MCOD	15	0.178 (0.039)	0.880	4.9
[c]P-450	15	0.360 (0.014)	0.940	2.6
[b]ALE	15	1.946 (0.342)	5.076	2.6
[b]EROD	15	0.042 (0.013)	0.066	1.6

Table 4.7 Effect of PB Induction on various Markers of Drug Metabolizing Capacity (Antipyrine-Tolbutamide Study)

PARAMETER	n	CONTROL	PB 80mg/kg INDUCTION	FOLD INCREASE
[a]CL(TOL)	16	0.13 (0.03)	0.38	2.9
[a]CL(AP)	16	2.22 (0.14)	5.27	2.4
[b]ECOD	16	0.419 (0.010)	1.904	4.5
[b]MCOD	16	0.257 (0.083)	1.029	4.0
[c]P-450	16	0.529 (0.113)	1.060	2.0
[b]ALE	16	1.617 (0.245)	3.426	2.1

n number of rats included in study
[a] ml/min/SRW
[b] nmole/min/mg microsomal protein
[c] nmole/mg microsomal protein

4.5.3.2.1 *Correlations between In-vivo Parameters*

In the first PB induction study, although correlations between the antipyrine metabolite formation clearances were good (r-range, 0.83-0.95), both CL(3H) and CL(N) correlated poorly with CL(TH) (r=0.62). Also CL(4H) (r=0.81) did not correlate better with CL(TH) than antipyrine (r=0.85). However CL(AP) correlated fairly well with both CL(N) (r=0.82) and CL(4H) (0.87). With the exception of correlations between CL(3H) and the other *in-vivo* parameters, all other *in-vivo* correlations were much poorer in the PB induction study than in the β-NF induction study (figure 4.21).

In the second PB induction study a strong correlation was observed between CL(AP) and CL(TOL) (0.89) (figure 4.22).

4.5.3.2.2 *Correlations between In-vitro Parameters*

Since both MCOD and ECOD activities are very sensitive to induction by PB, it was not surprising that these parameters correlated highly with each other (r=0.96). The correlations between P-450 content and both MCOD (r=0.86) and ECOD activities (r=0.90) were also high. The insensitivity of EROD activity to PB induction suggested that this would be a poor choice of marker for PB-inducible forms of cytochrome P-450. Indeed correlations between EROD and the other *in-vitro* parameters proved to be poor (r-range, 0.30 - 0.65). The correlations observed with ALB activity were also too low to be of a predictive value (r-range, 0.30 - 0.83). Similar trends were observed for the *in-vitro* data generated in the second study. Figure 4.23 depicts the correlations between ECOD activity and the other four *in-vitro* parameters.

4.5.3.2.3 *Correlations between In-vivo and In-vitro Parameters*

Of the *in-vivo* parameters determined in the first PB induction study, CL(AP) and CL(4H) produced markedly better correlations with the *in-*

vitro parameters. Since none of the antipyrine metabolite formation clearances produced considerably better correlations than CL(AP) itself, it was not considered necessary to collect metabolite data in the second study (figure 4.25).

The trends observed in the correlations between CL(AP) and the *in-vitro* parameters (figure 4.24) were also found in the parameters CL(TH) (figure 4.26) and CL(4H). The highest correlation was found between CL(AP) and P-450 content (r=0.81) followed by ECOD (r=0.78) and then MCOD activities (r=0.77). The *in-vitro* parameters, EROD and ALE, and the *in-vivo* parameters, CL(N) and CL(3H), consistently produced poor *in-vitro/in-vivo* correlations (figure 4.25).

Although slight differences were observed between the *in-vivo/in-vitro* correlations obtained for CL(AP) in the two different PB studies, the values obtained with CL(TOL) were consistently better (figure 4.27). The strongest correlations being observed between CL(TOL) and ECOD (r=0.94) and MCOD activities (r=0.89).

Table 4.8 Relationships Between and Within *In-vivo* and *In-vitro* markers of Drug Metabolizing Capacity Measured in control and PB induced Rats (Antipyrine-Theophylline Study, n = 15).

CL(TH)	CL(4H)	CL(N)	CL(3H)	EROD	ALE	P-450	MCOD	ECOD	
0.846	0.873	0.816	0.691	0.399	0.759	0.806	0.765	0.782	CL(AP)
	0.812	0.623	0.617	0.407	0.635	0.715	0.653	0.639	CL(TH)
		0.853	0.831	0.533	0.582	0.807	0.741	0.732	CL(4H)
			0.949	0.298	0.398	0.533	0.465	0.445	CL(N)
				0.281	0.237	0.430	0.331	0.314	CL(3H)
					0.301	0.650	0.478	0.571	EROD
						0.722	0.772	0.827	ALE
							0.862	0.903	P-450
								0.964	MCOD

Table 4.9 Relationships Between and Within *In-vivo* and *In-vitro* markers of Drug Metabolizing Capacity Measured in control and PB induced Rats (Antipyrine-Tolbutamide Study, n = 16)

Cl(AP)	ALE	P-450	MCOD	ECOD	
0.891	0.671	0.839	0.894	0.939	CL(TOL)
	0.719	0.720	0.845	0.858	CL(AP)
		0.564	0.720	0.765	ALE
			0.847	0.887	P-450
				0.952	MCOD

The values represented in these tables are correlation coefficients.
Critical values of r; $p < 0.001$; n = 16, 0.725; n = 15, 0.704
The following show correlation coefficients and their corresponding coefficients of determination (%)

r = 0.95, r^2 = 90%; r = 0.90, r^2 = 80%;
r = 0.84, r^2 = 70%; r = 0.77, r^2 = 60%

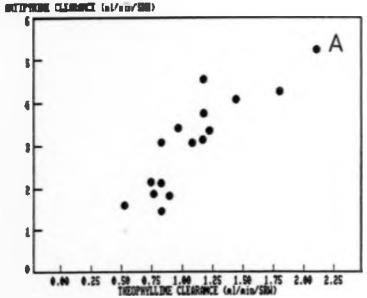
Figure 4.21 Correlations Between Theophylline Clearance and other *In-vivo* Markers of Drug Metabolizing Capacity in Control and PB induced Rats

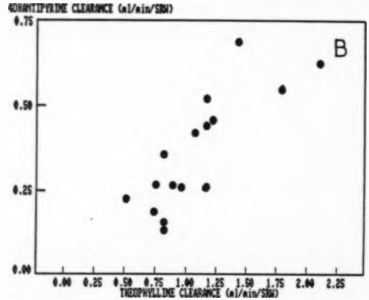
A - Antipyrine Clearance versus Theophylline Clearance

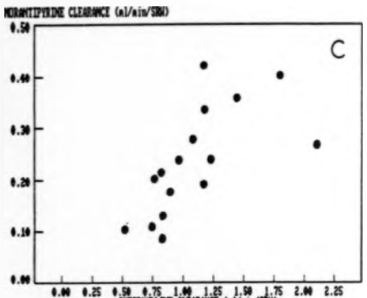
B - 4-OHAntipyrine Formation Clearance versus Theophylline Clearance

C - Norantipyrine Formation Clearance versus Theophylline Clearance

D - 3-OHMeAntipyrine Formation Clearance versus Theophylline Clearance


ANTIPYRINE CLEARANCE (ml/min/SBA)
A
THEOPHYLLINE CLEARANCE (ml/min/SBA)


4-AMINOPYRINE CLEARANCE (ml/min/SBA)
B
THEOPHYLLINE CLEARANCE (ml/min/SBA)


NORANTIPYRINE CLEARANCE (ml/min/SBA)
C
THEOPHYLLINE CLEARANCE (ml/min/SBA)

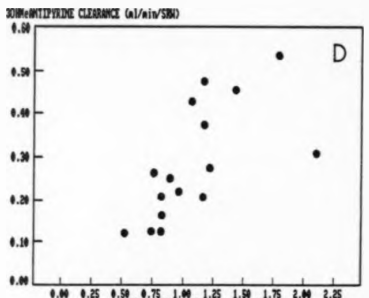

3-OH-ANTIPYRINE CLEARANCE (ml/min/SBA)
D
THEOPHYLLINE CLEARANCE (ml/min/SBA)

Figure 4.22 Correlation Between Tolbutamide and Antipyrine Clearances in Control and PB induced Rats

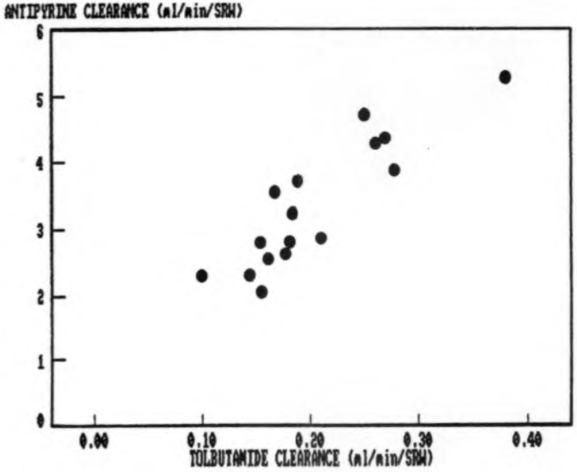

ANTIPYRINE CLEARANCE (ml/min/SRW)
6
5
4
3
2
1
0
0.00
0.10
0.20
0.30
0.40
TOLBUTAMIDE CLEARANCE (ml/min/SRW)

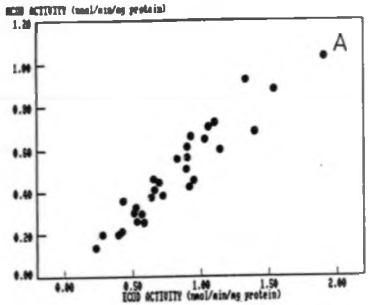
Figure 4.23 Correlations Between ECOD Activity and other *in-vitro* Markers of Drug Metabolizing Capacity in Control and PB induced Rats

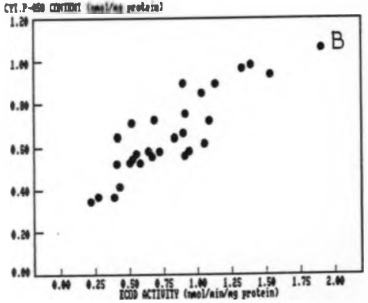
A - MCOD Activity versus ECOD Activity

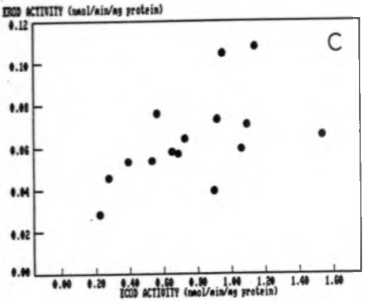
B - P-450 Content versus ECOD Activity

C - EROD Activity versus ECOD Activity

D - ALE Activity versus ECOD Activity


MCHD ACTIVITY (nmol/min/mg protein)
A
ECOD ACTIVITY (nmol/min/mg protein)


CYT.P-450 CONTENT (nmol/mg protein)
B
ECOD ACTIVITY (nmol/min/mg protein)


ECOD ACTIVITY (nmol/min/mg protein)
C
ECOD ACTIVITY (nmol/min/mg protein)

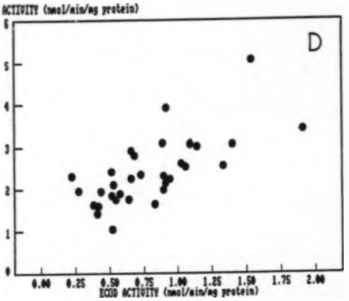

ALE ACTIVITY (nmol/min/mg protein)
D
ECOD ACTIVITY (nmol/min/mg protein)

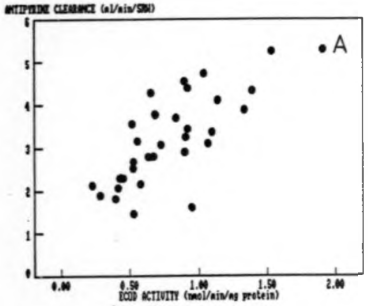
Figure 4.24 Correlations Between Antipyrine Clearance and various *In-vitro* Markers of Drug Metabolizing Capacity in Control and PB induced Rats

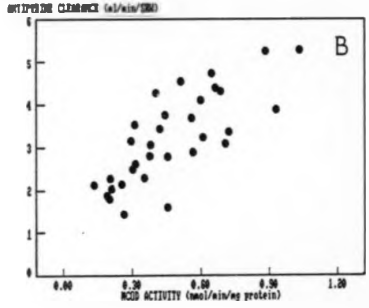
A - Antipyrine Clearance versus ECOD Activity

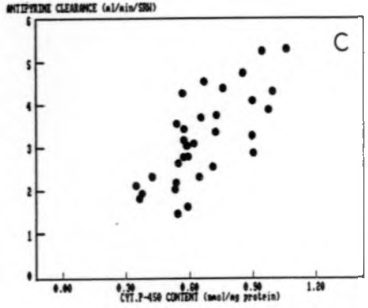
B - Antipyrine Clearance versus MCOD Activity

C - Antipyrine Clearance versus P-450 Content

D - Antipyrine Clearance versus ALE Activity


ANTIPYRINE CLEARANCE (ml/min/SKN)
A
ECOD ACTIVITY (nmol/min/mg protein)


ANTIPYRINE CLEARANCE (ml/min/SKN)
B
NCOD ACTIVITY (nmol/min/mg protein)


ANTIPYRINE CLEARANCE (ml/min/SKN)
C
CYT.P-450 CONTENT (nmol/mg protein)

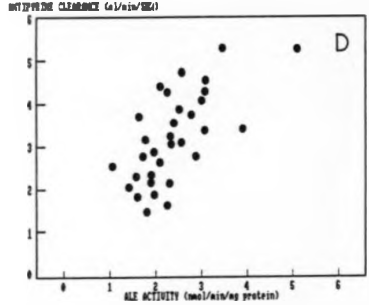

ANTIPYRINE CLEARANCE (ml/min/SKN)
D
ALE ACTIVITY (nmol/min/mg protein)

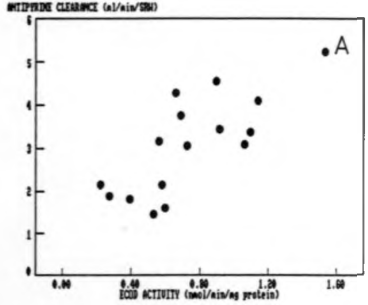
Figure 4.25 Correlations Between Antipyrine Formation Clearances and ECOD Activity in Control and PB Induced Rats

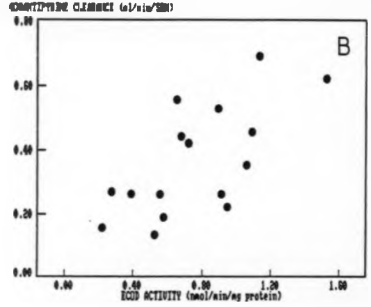
A - Antipyrine Clearance versus ECOD Activity

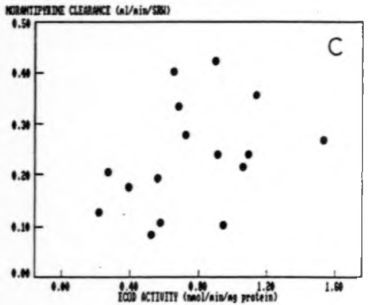
B - 4-OHAntipyrine Formation Clearance versus ECOD Activity

C - Norantipyrine Formation Clearance versus ECOD Activity

D - 3-OHMeAntipyrine Formation Clearance versus ECOD Activity


A
0.00
0.40
0.80
1.20
1.60
ECOD ACTIVITY (nmol/min/mg protein)


B
0.00
0.40
0.80
1.20
1.60
ECOD ACTIVITY (nmol/min/mg protein)


C
0.00
0.40
0.80
1.20
1.60
ECOD ACTIVITY (nmol/min/mg protein)

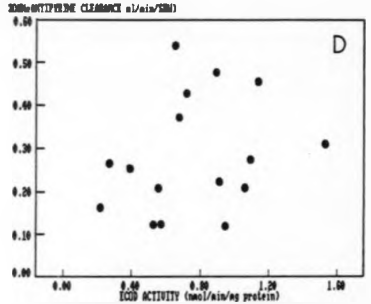

D
0.00
0.40
0.80
1.20
1.60
ECOD ACTIVITY (nmol/min/mg protein)

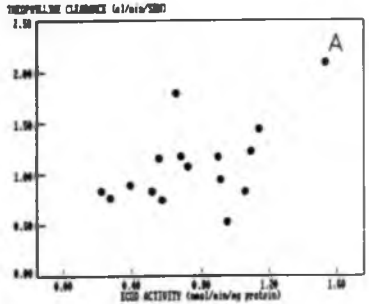
Figure 4.26 Correlations Between Theophylline Clearance and various *In-vitro* Markers of Drug Metabolizing Capacity in Control and PB induced Rats

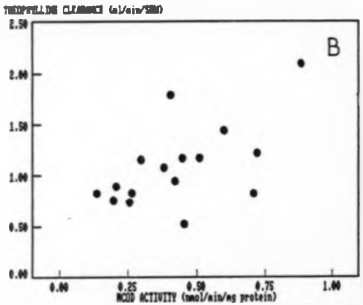
A - Theophylline Clearance versus ECOD Activity

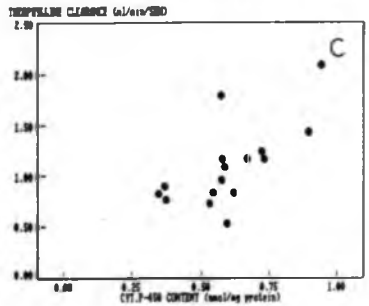
B - Theophylline Clearance versus MCOD Activity

C - Theophylline Clearance versus P-450 Content

D - Theophylline Clearance versus ALE Activity


A
2.50
2.00
1.50
1.00
0.50
0.00
0.00
0.50
1.00
1.50
ECOD ACTIVITY (nmol/min/mg protein)


B
2.50
2.00
1.50
1.00
0.50
0.00
0.00
0.25
0.50
0.75
1.00
MCOD ACTIVITY (nmol/min/mg protein)


C
2.50
2.00
1.50
1.00
0.50
0.00
0.00
0.25
0.50
0.75
1.00
CYT.P-450 CONTENT (nmol/mg protein)

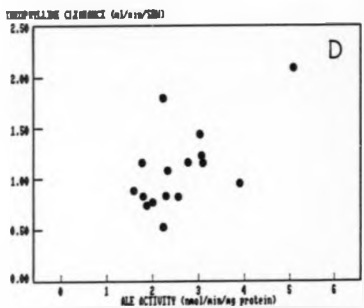

D
2.50
2.00
1.50
1.00
0.50
0.00
0
1
2
3
4
5
6
ALE ACTIVITY (nmol/min/mg protein)

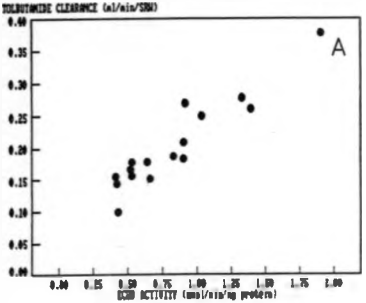
Figure 4.27 Correlations Between Tolbutamide Clearance and various *In-vitro* Markers of Drug Metabolizing Capacity in Control and PB induced Rats

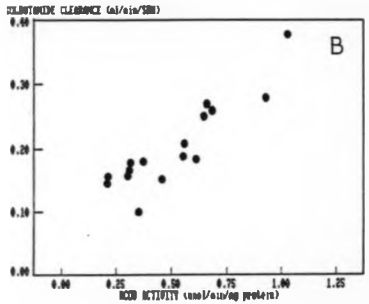
A - Tolbutamide Clearance versus ECOD Activity

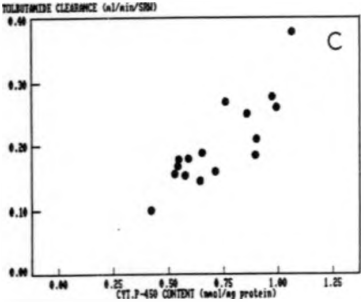
B - Tolbutamide Clearance versus MCOD Activity

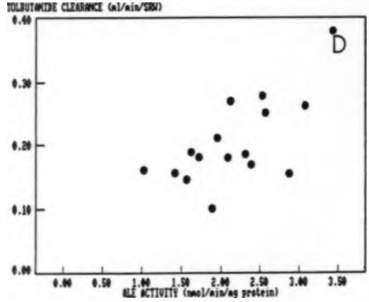
C - Tolbutamide Clearance versus P-450 Content

D - Tolbutamide Clearance versus ALE Activity


TOLBUTAMIDE CLEARANCE (ml/min/SRM)
A
0.40
0.35
0.30
0.25
0.20
0.15
0.10
0.05
0.00
0.00 0.25 0.50 0.75 1.00 1.25 1.50 1.75 2.00
BCHD ACTIVITY (nmol/min/mg protein)


TOLBUTAMIDE CLEARANCE (ml/min/SRM)
B
0.40
0.30
0.20
0.10
0.00
0.00 0.25 0.50 0.75 1.00 1.25
BCHD ACTIVITY (nmol/min/mg protein)


TOLBUTAMIDE CLEARANCE (ml/min/SRM)
C
0.40
0.30
0.20
0.10
0.00
0.00 0.25 0.50 0.75 1.00 1.25
CYT.P-450 CONTENT (nmol/mg protein)


TOLBUTAMIDE CLEARANCE (ml/min/SRM)
D
0.40
0.30
0.20
0.10
0.00
0.00 0.50 1.00 1.50 2.00 2.50 3.00 3.50
ALE ACTIVITY (nmol/min/mg protein)

4.5 CONCLUSIONS

Previous attempts to compare *in-vitro* and *in-vivo* markers of drug metabolizing ability of an individual were relatively unsuccessful, however the present studies suggest this to be due mainly to disregard of the multiplicity of the cytochrome P-450 system. Therefore use of a number of different markers with specificity for particular isoenzymes of cytochrome P-450 may be of more value than attempting to correlate the metabolism of a large number of drugs to one marker such as antipyrine. Other workers (Taunissen *et al.*, 1986; Van der Graaff *et al.*, 1983b) have investigated another approach, using antipyrine which produces several metabolites, since different forms of cytochrome P-450 may mediate the different pathways (Rhodes and Houston, 1983b; Breimer, 1983). Indeed the measurement of antipyrine formation clearances in animals and man has shown that these metabolites can be selectively induced or inhibited by other compounds (table 1.2).

A comprehensive study by Taunissen *et al.* (1986) using a rat model, produced a correlation matrix between clearances of antipyrine and theophylline and formation clearances of their respective metabolites. A wide range in drug metabolizing ability was achieved in these rats using the inducer 3-MC which, like β-NF, is specific for cytochromes P450I. Since the correlations between theophylline and antipyrine were only improved by use of antipyrine metabolite data not by use of theophylline metabolite data, only the former were determined in the present study.

The β-NF induction studies presented here, with respect to correlations between *in-vivo* parameters, confirmed the findings of Taunissen *et al.* (1986) that CL(TH) and CL(4H) are highly correlated, and confirmed that CL(4H) was a better marker of P450I induction than CL(AP).

The PB induction studies indicated that although theophylline metabolism was susceptible to such an inducer, the induction effect was

much smaller than that produced by β-NF. Correlations between CL(TH) and other markers of drug metabolizing capacity were significant but of no predictive value. CL(AP) correlations were better than those of CL(TH) but still were too low to be useful for the prediction of drug metabolizing capacity. No improvement was observed using metabolite formation clearances instead of antipyrine total clearance. The lack of selectivity of PB towards induction of these particular metabolic pathways observed in this study confirmed the previous observations of Tounisssen *et al.* (1983b) and Rhodes and Houston (1983b). The strongest correlations were observed with CL(TOL). This, together with the fact that β-NF administration produced no effect on the kinetics of tolbutamide, confirmsits suitability as a specific marker of PB induction in the rat.

Various groups have investigated the specificity of different assays towards induction. MCOD activity is specific for PB induction (Paterson *et al.*, 1984), HROD activity is specific for β-NF induction (Burke and Mayer, 1974, 1975, 1983; Burke *et al.*, 1985; Lubet *et al.*, 1985) ALE activity is inducible by PB but inhibited by 3-MC (Wolff *et al.*, 1979; Van Cantfort *et al.*, 1983) and ECOD activity (Ullrich *et al.*, 1973) and P-450 content are both sensitive to induction by PB and β-NF. In order to investigate whether the use of substrates specific for particular induced forms of cytochrome P-450 might improve correlations with *in-vivo* probe compounds, these studies were undertaken.

In the β-NF studies, the lack of any relationship between *in-vivo* parameters and MCOD and ALE activity confirmed that the choice of *in-vitro* substrate is indeed important. However although it was expected that HROD activity would provide the best correlations in fact the *in-vivo/in-vitro* correlations produced by ECOD activity, were slightly better than those produced by HROD activity. This may be due to the fact that although cytochromes P450I are important in the metabolism of

antipyrine, theophylline and ethoxycoumarin, other PB-inducible forms of P-450 are also involved.

In the PB studies, the correlations between *in-vivo* markers and both MCOD and ECOD activities were consistently better than with any of the other *in-vitro* markers. Although ECOD activity proved to correlate more strongly with CL(TOL) than MCOD activity, correlations between these two *in-vitro* markers and other *in-vitro* and *in-vivo* markers were very similar.

These studies supported the idea that careful selection of markers of drug metabolizing ability can improve correlations between and within measured *in-vivo* and *in-vitro* parameters. A number of factors related to the experimental design also contributed to the success of these correlation studies. The same rats were used for both the *in-vitro* and *in-vivo* measurements and markers were coadministered to minimise inter- and intra-individual variability. In conclusion these studies illustrate that correlations can be established under the careful controlled conditions possible in animal experimentation. These also indicate that much of the lack of correlation in the literature is a function of poor experimental planning rather than a lack of association between *in-vitro* and *in-vivo* markers.

CHAPTER 5

ASSESSMENT OF INHIBITORY POTENCY USING *IN-VITRO* AND *IN-VIVO* METHODS

5.1 INTRODUCTION

5.1.1 The Inhibition Phenomenon

Classical enzyme kinetic studies, utilising purified enzymes, have limited relevance to *in-vitro* studies of cytochrome P-450 inhibition, since the multiplicity of enzymes present and the diversity of response to inhibition creates inhibition profiles which bear little relationship to those classically described. Despite these drawbacks, inhibition studies of *in-vitro* drug metabolism can provide an indication of inhibitory potency.

Effective inhibitors of drug metabolism *in-vitro* do not always produce *in-vivo* inhibition. In order for one drug to inhibit the metabolism of another, it must accumulate at the site of metabolism in sufficient quantities as to compete with the metabolism of the drug in question. Exceptions to this are for compounds which irreversibly bind to cytochrome P-450 or destroy the haemoprotein. Factors which influence the concentration of drugs at these sites include tissue or protein binding and the rates of excretion or metabolism. If the inhibition is also selective, then the enzymes involved in the metabolism of the co-administered drug may not be affected. *In-vivo* inhibition studies are further complicated by any influences the inhibitor may have on the volume of distribution and biliary and renal excretion of the interactive compound.

Many compounds can inhibit drug metabolism when administered in single dose. Few, however, can sustain the inhibition during chronic administration, since transient inhibition is often followed by induction. The inhibitory potency observed for a compound *in-vivo* is therefore highly dependent upon the experimental design.

5.1.1.1 *Types of Inhibitor*

Since the mixed function oxidase system consists of a large number of different isoenzymes, it is difficult to describe its inhibitors by classical methods. Testa and Jenner (1981), however, reviewed the literature on inhibitors of drug metabolism and designated various groups of inhibitors:-

Irreversible inhibitors covalently bind to the haem or apoprotein of cytochrome P-450 either directly or indirectly (requiring metabolism to a reactive intermediate). In order to learn more about the inhibition mechanism, Halpert and co-workers (1986) investigated the inhibitory potency of a series of such compounds. An example of such an inhibitor is chloramphenicol.

Reversible inhibitors bind directly or indirectly (requiring activation) to oxidised and reduced P-450 in a ligand type fashion. A variety of imidazoles, such as cimetidine, are able to bind directly, methylenedioxyphenyls bind following activation.

A number of metal ions such as cobalt and organic agents such as ethionine and amantadine are able to alter the synthesis or degradation of cytochrome P-450.

5.1.1.2 *Mechanisms of Reversible Inhibition*

The type of inhibition observed for an inhibitor depends on its spectral binding type, the substrate involved in the interaction, the relative times at which the inhibitor and substrate are added to the system, the animal species and the complement of cytochromes P-450 (Gillette *et al.*, 1973). As a consequence, depending on the prevailing conditions, competitive, non-competitive and/or mixed inhibition may be observed for each inhibitor. Generally, reversible inhibitors may bind to the reduced and/or the oxidised forms of cytochrome P-450. Inhibitors may act as alternative substrates and thus compete with other substances for the

substrate binding site on the enzyme, or may bind as ligands to cytochrome P-450 thus preventing the reduction of ferricytochrome P-450 and/or the binding of oxygen. Information about the type of interaction produced by a compound may be derived from spectral binding studies since alternative substrates produce a type I spectral interaction with cytochrome P-450, whereas ligand binding is characterised by a type II spectral interaction. An intermediate situation, observed by production of a reverse type I spectrum, indicates that a substance is a weak ligand of cytochrome P-450 (Testa and Jenner, 1981).

Gillette *et al.* (1973) have formulated three kinetic models for the inhibition of drug metabolism by substances acting at different stages in drug metabolism.

i) The first model holds when the substrate can only react with the oxidized form of cytochrome P-450 to form a complex which is reduced by cytochrome P-450 reductase. The substrate cannot be removed from the cytochrome after it has been reduced so an inhibitor can only attack at the first step, the initial binding to the oxidized cytochrome. This model predicts pure competition at the initial binding site and linear Lineweaver-Burk plots. Both type I and type II inhibitors should inhibit the metabolism of type I substrates by a competitive mechanism.

ii) In the second model the substrate can combine with, and dissociate from, every state of cytochrome P-450, oxidized, reduced, oxygenated and intermediate states. This is more complicated with competitive inhibition only occurring in certain situations.

iii) The third model, by making certain assumptions, is a simplification of the second. It is assumed that in air, the proportion of reduced cytochrome P-450 is negligible and the proportion of the oxygenated cytochrome P-450-substrate complex should be small. This model produces rate equations which yield curved Lineweaver-Burk plots and mixed forms of inhibition.

Models two and three predict that competition can only be expected for type I substrates and inhibitors when secondary mechanisms are considered. Clearly since a number of inhibitors can act both competitively and non-competitively these models are still oversimplifications (Burgschat and Netter, 1977).

5.1.1.3 *Inhibition of Drug Metabolism by Imidazoles*

A number of imidazole derivatives are strong ligands of the haem iron of cytochrome P-450 and in binding experiments yield type II binding spectra (Wilkinson *et al.*, 1972). These have the ability to bind to both oxidized and reduced forms of cytochrome P-450 and therefore may interfere with more than one step in metabolism. They may compete with binding of substrate to ferricytochrome P-450 and binding of oxygen to the substrate-ferrocytochrome P-450 complex. This results in strong inhibitory activity. Imidazole binding to cytochrome P-450 depends on the combination of lipophilicity (Wilkinson and Netnarski, 1974) and structural features such as polarity, rigidity and size of various parts of the molecule. The 2- and 4,5- substituents of 2-substituted and 4,5-disubstituted imidazoles sterically hinder one or both of the nitrogen heterocycles. Since this is reflected in a reduction in inhibitory activity, at least one of the imidazole nitrogens must be unhindered for strong binding and potent inhibitory activity (Rogerson *et al.*, 1977).

Particular interest has been directed towards investigation of the inhibitory potency of the imidazole derivative, cimetidine. Inhibition of drug metabolism by cimetidine has been demonstrated both *in-vitro* and *in-vivo* (Mitchell *et al.*, 1984; Palkonen and Puuronen, 1980; Spaeg Jr. *et al.*, 1982). Somogyi and Gugler (1982, 1983) indicated that the concentration of cimetidine for *in-vitro* inhibition is 10 to 100 times greater than that needed *in-vivo*. Reilly *et al.*, (1983) suggested that the inhibition of cytochrome P-450 by cimetidine could involve different

isoenzymes. Scatchard analysis of the spectral binding data indicated that there were two binding sites for cimetidine, a high-affinity, low-capacity site (dissociation constant (K_s)-2.6μM) and a low-affinity, high-capacity site (K_s-104μM). It was pointed out, however,that the curvilinear nature of the Scatchard plot could result from either heterogeneity or negative cooperativity of the cytochrome P-450 binding sites. Further equilibrium partition studies (Reilly *et al.*, 1983) reinforced the idea that cimetidine has two distinct binding sites (K_{s_1}-6.3μM and K_{s_2}-104μM). Since chronic cimetidine administration produces concentrations which do not exceed 6μM (Somogyi and Gugler, 1983) it is possible that the inhibition of drug metabolism observed *in-vivo* is due to an interaction with the high-affinity site, whereas the interaction studied *in-vitro* may be between cimetidine and a low-affinity site (Reilly *et al.*, 1983). This is suggested because the inhibitor constant values derived from *in-vitro* studies are in the range 0.5 to 10mM (Drew *et al.*, 1981; Pelkonen and Puuronen, 1980; Puuronen and Pelkonen, 1979; Rendic *et al.*, 1979; Serlin *et al.*, 1980). These data indicate the value of using a variety of *in-vitro* and *in-vivo* methods to investigate the inhibition phenomenon.

5.1.2 Ketoconazole

Ketoconazole is a 1-substituted imidazole and as such might be expected to inhibit oxidative drug metabolism (Wilkinson *et al.*, 1974). *In-vitro* studies have indicated that ketoconazole binds to microsomes in a similar manner to cimetidine (Brown *et al.*, 1983; Meredith *et al.*, 1983) and can indeed inhibit a number of cytochrome P-450 dependent enzyme activities (Brown *et al* 1985; Loose *et al* 1983; Meredith *et al.* 1985; Sheets and Mason, 1984). *In-vivo* inhibition data is, however, scant and contradictory (Daneshmend, 1982; Ferguson *et al* 1982; Meredith *et al* 1985; Niemegeers *et al* 1981). Ketoconazole prolongs methohexitone

sleep time and exaggerates acenocoumarol anticoagulation in the rat but these changes occur only with doses well above the accepted therapeutic range (Niemegeers *et al.*, 1981). Several clinical reports have suggested that ketoconazole may inhibit hepatic oxidative drug metabolism in man (Dieperink *et al.*, 1982; Ferguson *et al.*, 1982; Brown *et al.*, 1985), criticism has however been made of the first two studies in that factors other than inhibition of drug metabolism may be involved in the interactions (Daneshmand, 1982).

Ketoconazole is a broad spectrum antifungal agent used to treat a wide variety of superficial and systemic mycoses (Borelli *et al.*, 1979; Gascoigne *et al.*, 1981; Graybill and Drutz, 1980; Heel *et al.*, 1982; Odds *et al.*, 1980). Its greater water solubility and better gastro-intestinal absorption than other imidazole antifungals make it more suitable for oral administration.

Whilst ketoconazole was primarily introduced as an antifungal agent, a large number of other potential uses have subsequently been identified. Ketoconazole-induced inhibition of steroid biosynthesis, for example, has been used therapeutically to treat hormone overproduction in conditions such as Cushing's syndrome (Contreras, 1985; Engelhardt *et al.*, 1983; Shepherd *et al.*, 1985; Sonino *et al.*, 1985) and precocious puberty (Holland *et al.*, 1985) as well as to reduce androgen levels in patients with prostatic cancer (Trachtenberg *et al.*, 1983; Trachtenberg 1984; Trachtenberg and Pont, 1984). High dose ketoconazole therapy in prostatic cancer patients also produced a 27% reduction in total serum cholesterol levels without affecting serum triglyceride levels (Kraemer and Pont, 1986). Although further studies are required, in particular into the effects of elevated levels of lanosterol resulting from this inhibition, ketoconazole may have use in hypercholesterolaemia (Kraemer and Pont, 1986).

Ketoconazole has been demonstrated to be a specific and orally active inhibitor of 5-lipoxygenase activity (Beetens *et al.*, 1986). This enzyme is involved in the transformation of arachidonic acid to leukotrienes which are thought to be important mediators of allergic and anaphylactic reactions and of inflammation (Samuelsson, 1983). Ketoconazole may therefore have potential use as a tool for the investigation of leukotriene biosynthesis regulation and as a drug for the treatment of disease states associated with hypersensitivity reactions and inflammation (Beetens et al., 1986).

Beach et al. (1986) have investigated the effect of ketoconazole on sterol biosynthesis by *Trypanosoma cruzi* epimastigotes. Studies in mice (McCabe *et al.*, 1984; Raether and Seidenath, 1984) and mouse peritoneal macrophages and fibroblasts (McCabe *et al.*, 1984) showed ketoconazole to be effective against *Tr. cruzi* amastigote infections and suggest that ketoconazole should also be tested against American trypanosomiasis (Chaga's disease).

In-vitro studies have shown that ketoconazole has antiviral activity in its own right and exhibits synergy with adenine arabinoside against the Herpes simplex virus (Pottage *et al.*, 1986).

5.1.2.1 *Mode of Antifungal Action of Ketoconazole*

The primary mode of action of ketoconazole appears to be disorganization of the fungal plasma membrane. Membrane function is disturbed after application of low concentrations which lead to changes in membrane permeability accompanied by alterations in specific activity of membrane-associated enzymes. These finally result in inhibition or death of the fungal cell. Action relates to ketoconazole-induced interference with ergosterol synthesis. Ergosterol is an important component of fungal membranes where it enhances the membrane stability through complexation with phospholipids and stabilization of phospholipid

domains. Lack of ergosterol as well as accumulation of sterol precursors, consequently leads to disruption of membranes (Berg *et al.*, 1986).

After addition of ketoconazole to suspension cultures of human pathogens, 24-methylene-dihydrolanosterol, a precursor of ergosterol, accumulates, indicating that the subsequent step in sterol synthesis is inhibited. (i.e. the oxidative demethylation at C-14). The enzyme complex responsible for the oxidative removal involves a cytochrome P-450 system and a direct type II interaction between the prosthetic group of P-450 (the Fe-porphyrine complex) has been proven (Berg *et al.*, 1986).

5.1.2.2 ***Toxicological studies on ketoconazole***

Toxicological data generated by Janssen Pharmaceutica Research laboratories is published (Heel, 1982). As expected, animal LD_{50} values are considerably higher than the dose required to produce therapeutic effects.

5.1.2.2.1 *Animal Toxicity*

Chronic animal toxicity studies conducted on ketoconazole, in rats, by Janssen Pharmaceutica Research Laboratories, produced only minor toxic effects at daily doses of 20 to 40mg/Kg. These were accentuated at higher doses (80 to 160mg/Kg). Signs of toxicity included reductions in food consumption and weight gain. Gross changes in the liver, kidneys, adrenals and ovaries were observed, as reflected by increased serum sodium and blood urea nitrogen concentrations and reduced serum potassium, urinary creatinine and urinary specific gravity.

Chronic toxicity studies in Beagle dogs indicated that the liver was the primary target for toxicity. A daily dose of 40mg/Kg (one year) produced emesis and a reduction in food consumption and weight gain. Liver weight increased with increased serum concentrations of glutamic-

pyruvic transaminase (SGPT) and alkaline phosphatase. There was also evidence of lipofuscin formation and deposition. Higher doses (60mg/Kg; 20 weeks) produced more marked changes in SGPT and alkaline phosphatase as well as increased liver but reduced thymus weight. All changes were reversible on cessation of treatment (Heel, 1982).

Long-term treatment with ketoconazole has been shown to impair the fertility of male rats by decreasing sperm number and motility, and increasing the number of abnormal sperm (Vawda and Davies, 1986).

5.1.2.2.2 *Toxicity observed during Clinical Usage of Ketoconazole*

Ketoconazole was received enthusiastically, when marketed in 1979, as an antifungal which could be administered via the oral route. As its clinical use increased, reports of adverse hepatic reactions appeared (Heiberg and Svejgard, 1981; Janssen and Symoens, 1983; Lewis *et al.*, 1984). Janssen and Symoens (1983) observed that, in a small proportion of patients (5 to 10%), there were raised levels of hepatocellular enzymes. No other symptoms were noted and post-treatment levels returned to normal. Symptoms of hepatitis were recorded in an estimated 1 of 12,000-15,000 patients treated with ketoconazole (Janssen and Symoens, 1983; Lewis *et al.*, 1984). These patients presented with jaundice, anorexia, nausea and vomiting, as well as raised levels of hepatic enzymes. These effects also reversed when treatment ceased. Lewis *et al.* (1984) and Barcoff *et al.* (1985) believed that the hepatotoxicity was most likely to be due to a direct or metabolite-mediated toxicity. The risk of developing hepatotoxicity is greater in patients on long-term treatment (greater than 14 d) (Cauwenbergh, 1986).

5.1.2.2.3 *Side Effects observed during Ketoconazole Administration*

Alterations in liver function test results have occurred in patients taking ketoconazole. These changes are usually transient but a few cases

of hepatitis have been reported (Horsburgh *et al.*, 1982).

In rare cases anaphylatic reactions have been observed after the first dose. Hypersensitivity reactions including urticaria and angio-oedema have also been reported. The most commonly observed side-effects are anorexia, mild nausea and/or occasional vomiting. This occurs in 3-21% of patients on oral ketoconazole therapy (Heel *et al.*, 1982; Diamuka *et al.*, 1983) together with rash, urticaria, pruritis and headaches.

Ketoconazole can inhibit steroid synthesis observed occasionally as the development of gynecomastia (DeFelice *et al.*, 1981). Reports by Rollman *et al.* (1985) and Kraemer and Pont (1986) suggest that ketoconazole also has a transient inhibitory effect on serum lipid levels.

5.1.2.3 ***Metabolism of Ketoconazole***

Information about the metabolites of ketoconazole is confined to data produced by Gascoigne *et al.* (1981). In dogs and rats it is extensively metabolized to a large number of inactive metabolites. The main metabolic pathways include oxidation and subsequent scission and degradation of the imidazole ring, scission and degradation of the piperazine ring, scission of the dioxolane ring and oxidative O-dealkylation.

In rats more than 95% of an orally administered dose (10mg/Kg) is excreted in 4 days. The amount excreted in the urine is gender-dependent (17% for males, 5% for females) but only a small amount (< 0.1%) is unchanged drug. Biliary excretion accounts for 60% of the dose in both male and female rats. Only a small proportion of the dose (4-6%) is excreted unchanged in the faeces.

In female dogs, of the 92% of an orally administered dose (10mg/Kg) excreted in 7 days, 9% is eliminated in the urine and 83% in the faeces.

A much higher proportion is recovered unchanged in dog faeces (70%), possibly due to poorer absorption.

In human, as in animal studies, metabolism of ketoconazole is extensive. With the exception of scission of the dioxolane ring metabolic pathways are similar in animals and man. There is also a possibilty of aromatic hydroxylation in man. Four days after an oral dose (2.5mg/Kg) of ketoconazole 70% was excreted, 57% in faeces and 13% in urine. The faeces contained 20-65% unchanged drug whereas this was only 2-3% of that excreted in the urine.

5.1.2.4 *Pharmacokinetics of Ketoconazole*

5.1.2.4.1 *In Animals*

Absorption of ketoconazole from the gut is more rapid in rats and guinea- pigs (max at 0.25 to 1 h) than in rabbits and dogs (max at 1 to 2 h) Following a dose of 10mg/Kg, peak concentrations were lowest in rabbits (0.9µg/ml) and highest in rats (12.9µg/ml) (Heykants *et al.*, 1978). The extent of absorption was approximately the same in fasted and nonfasted rabbits but peak concentrations were lower and occurred slightly sooner in fasted animals. In rats a gender-related difference in the rate of absorption was seen, absorption occurring more rapidly in males (peak at 0.25-1 h and 2-4 h in males and females respectively). AUC varied more than 10-fold between species given the same dose, the largest AUCs occurring in dogs and rats (Heel, 1982). The half-lives observed were lowest for guinea-pigs (0.7 h) and highest for the dog (2.8 h) (Heykants *et al.*, 1978).

A chronic dosage study in dogs receiving daily oral doses of 2.5, 10 or 40mg/kg ketoconazole, indicated that drug levels were greater when analysed after prolonged treatment than after the first dose and were dose-related (Van Cutsem and Thienpont, 1977).

5.1.2.4.2 *In Man*

In man a 200mg oral dose produced peak plasma concentrations of 3.0-4.5 µg/ml, 1 to 2 h post-administration (Huang *et al.*, 1986; Gascoigne *et al.*, 1981).

The disposition of ketoconazole is thought to follow a multi-exponential pattern with distribution equilibrium not being reached until about 8 to12 h post-administration (Gascoigne *et al.*, 1981; Huang *et al.*, 1986). Other studies (Daneshmand *et al.*, 1981, 1983, 1984; Maksymiuk *et al.*, 1982) have used a single compartment model in man. This may have been so due to less sensitive analytical methods or to shorter blood sampling schedules. Huang *et al.* (1986) found that on reaching C_{max}, the plasma decline was biphasic with a mean half-life of 1.6 ± 0.5 h during the first 8 to 12 h and a mean half-life of 7.6 ± 3.5 h there after. This was similar to the data previously generated by Gascoigne *et al.* (1981). The mean $AUC_{12 \rightarrow \infty}$ contributed more than 95% of the total which suggests that accumulation of ketoconazole on long-term administration is minimal (Gibaldi and Perrier, 1982). There was wide intersubject variability in the profiles (Huang *et al.*, 1986; Daneshmand *et al.*, 1981) due to inter-individual differences in absorption and distribution.

Dose dependency studies (Huang *et al.*, 1986; Gascoigne *et al.*, 1981; Daneshmand and Warnock, 1983; Brass *et al.*, 1982; Daneshmand *et al.*, 1981; 1984) suggested that in the former study dose-dependent kinetics were in operation. Dose-normalized Cp-t plots were not superimposable and the increase in AUC was not proportional to the increase in dose. The concentration-time plots also curved inwards at higher doses. These phenomena may be due to first pass metabolism in the liver or gut becoming saturated at higher doses. Dose-dependent kinetics have also been observed previously (Gascoigne *et al.*, 1981; Daneshmand *et al.*, 1984). Ketoconazole is widely distributed and extensively bound (99% at

a concentration of 1µg/ml *in vitro* in human plasma and serum (Heel *et al.*, 1982; Johnson *et al.*, 1985).

Clinically, ketoconazole is recommended to be administered immediately before or with a meal since this produces higher and more consistent levels. Ketoconazole is a base and in the acidity of the stomach it forms the hydrochloride salt. Hence it is thought that administration of ketoconazole on an empty stomach or with agents reducing gastric secretion such as cimetidine or antacids markedly reduces its oral absorption (Van der Meer *et al.*, 1980; Symoens *et al.*, 1980). Studies by Dismukes *et al.* (1983) and Daneshmend *et al.* (1981) confirm this supposition, studies by Brass *et al.* (1982) and Daneshmend *et al.* (1984) found that food decreased and increased absorption respectively but this effect was not significant. Yet another study (Mannisto *et al.*, 1982) found that breakfast significantly reduced the absorption of ketoconazole.

5.1.2.5 ***Inhibition of Drug Metabolism by Ketoconazole***

The mode of action of ketoconazole for its antifungal activity and for most of its other uses, is as an inhibitor of steroid synthesis. Although inhibition of cytochrome P-450 is more specific for fungal than the mammalian forms, studies have shown that when used at high dose inhibition of mammalian P-450 occurs (Loose *et al* 1983).

Experiments *in-vitro* have shown ketoconazole to be a potent inhibitor of N-demethylation in control rat microsomes (Meredith *et al.*, 1985) and microsomes from rats pretreated with PB or PCN (Sheets and Mason, 1984), and of 7-ethoxyresorufin O-deethylation in microsomes in rats pretreated with 3,6-benzoflavone (Sheets and Mason, 1984). Potent inhibition of nitroanisole O-demethylation in microsomes from rats pretreated with PB has also been observed (Lavrijsen *et al.*, 1987). Meredith *et al.* (1985) found that ketoconazole was a more specific inhibitor towards 3-MC

inducible P-450a. Lavrijsen *et al.* (1987), however, found it to have equipotent inhibitory activity against PB-and 3-MC-inducible forms of cytochrome P-450.

Ketoconazole produces a type II difference spectrum when incubated with microsomal suspensions (Lavrijsen *et al.*, 1987). This suggests that ketoconazole forms a ferrihaemochrome with cytochrome P-450 (Schenkman 1967), probably through an interaction of one of the nitrogens of the imidazole moiety (-3-N), with the ferric form of the haem group as a sixth ligand (Schenkman *et al.*, 1981). Studies by Sheet and Mason (1984) demonstrated that ketoconazole does not act through inhibition of cytochrome P-450 reductase or through formation of an inhibitory complex. Lavrijsen *et al.* (1987) have also suggested that binding may occur with a hydrophobic moiety on the apoprotein with additional binding sites on the microsomal phospholipid. This latter might result in conformational changes in the protein (Lenk, 1976). Most enzyme kinetic studies suggest that the interactions are mainly of a 'mixed' type of inhibition (Lavrijsen *et al.*, 1987; Meredith *et al.*, 1985). Ketoconazole binding studies indicate differences according to the cytochrome P-450 complement. Binding to control rat microsomes and to microsomes from 3-MC induced rats is biphasic, suggesting the existence of at least two binding sites. Binding to microsomes from PB-induced rats is linear and suggests the presence of cytochromes with a high affinity for ketoconazole (Lavrijsen *et al.*, 1987).

In-vivo inhibition studies are less conclusive in that at a high doses (50mg/Kg; ip) ketoconazole inhibits both aminopyrine (56% inhibition) and caffeine demethylation (83% inhibition; Meredith *et al.*, 1985). The inhibition is dose-dependent in the rat and sustained on chronic administration (7 d). Jiritano *et al.* (1986), however, found that chronic administration of ketoconazole (80mg/Kg; po; 15 d) did not affect the pharmacokinetics of either caffeine or phenytoin. Niemegeers

et al. (1981) found that male and female rats showed prolongation of methohexitone hypnosis at ED_{50} values of 97.0 and 30.4mg/Kg respectively and that the lowest effective dose for prolongation of prothrombin times was 50mg/Kg. These data suggest that ketoconazole administration in-vivo produces only weak inhibition. Evidence for inhibition of drug metabolism during ketoconazole therapy in man is also conflicting. Ketoconazole has no effect on antipyrine clearance during chronic administration (Daneshmend, 1982; Blyden *et al.*, 1986) but might potentiate the effects of oral anticoagulants (Smith, 1984; Braas *et al.* 1982). In two patients, an increase in cyclosporin levels was observed after the start of ketoconazole therapy (Ferguson *et al.*, 1982; Dieperink and Moller, 1982). Although this was suggested to be due to inhibition of drug metabolism, other factors may have been involved in the interaction (White *et al.*, 1984; Daneshmend, 1982). Ketoconazole has no effect on theophylline pharmacokinetics, but reduced the plasma clearance of chlordiazepoxide. After a single dose, a 20% decrease in clearance and a 26% reduction in volume of distribution has been reported. The changes were unrelated to dose. On chronic dosing, chlordiazepoxide clearance was decreased by 38% and was associated with reduced concentrations of its oxidative metabolite N-desmethylchlordiazepoxide (Brown *et al.*, 1985). Glynn *et al.*, (1986) demonstrated that ketoconazole (200mg/day for 6 days) decreased methylprednisolone clearance (60%), the terminal phase slope (37%), and volume of distribution (23%). They suggested that this was due to an inhibition of drug metabolism (Engelhard *et al.*, 1984; Daneshmend *et al.*, 1983).

Although there is strong evidence that ketoconazole may inhibit the metabolism of other compounds, many facets of this inhibition are unclear. Consequently it was considered to be a suitable compound for

the assessment of inhibitory potency both *in-vivo* and *in-vitro* in this study.

5.2 *IN-VITRO* INHIBITION OF DRUG METABOLISM

5.2.1 Introduction

In-vitro inhibition of drug metabolism by ketoconazole is well established. There have, however, been few studies which attempt to relate its inhibitory potency with that of other inhibitors. The aim of these studies was to compare the inhibitory potencies of ketoconazole with that of cimetidine, another imidazole derivative, known to be an inhibitor of both *in-vitro* and *in-vivo* metabolism (Speag Jr. *et al.*, 1982; Mitchell *et al.*, 1984).

5.2.2 Experimental Design

Rat liver microsomes were prepared (section 2.3.1) from untreated (control) rats (n=3) and rats treated (ip) with either 3 daily doses of β-NF (n=3, 100mg/Kg) or PB (n=3, 80mg/Kg). The microsomes in each group were pooled and stored at -70°C until required. These microsomal preparations were assayed for microsomal protein (section 2.3.2). Table 5.1 summarises details of the inhibition studies. ECOD, MCOD and EROD assays were carried out as described previously (section 2.3.4, 2.3.5 and 2.3.6) except that various concentrations (0 - 50μM, ketoconazole; 0 - 5mM, cimetidine) of the inhibitory agents were added in dimethylformamide (10μl) 10 min before starting the reaction. Experiments were performed in triplicate at each substrate concentration. In order to determine the inhibitor concentrations producing 50% inhibition (IC_{50}) for each substrate concentration, data were plotted as enzyme activity (% of control activity following addition of 10μl of DMF) against log inhibitor concentration. Dixon plots (1/enzyme activity against inhibitor concentration) enabled estimation of the inhibition constant (K_i). The points of intersection of each line were determined and the X-coordinates averaged in order to calculate the K_i and its standard deviation.

Table 5.1 Summary of the *In-vitro* Inhibition Studies Indicating which Microsomal Preparations were Employed for each Enzyme Assay

Microsomes	ECOD activity	MCOD activity	EROD activity
KETOCONAZOLE STUDY			
Control	YES	YES	NO
CIMETIDINE STUDY			
Control	YES	YES	NO
PB-induced	YES	YES	NO
β-NF-induced	YES	NO	YES

5.2.3 Results and Discussion

Tables 5.1 and 5.2 summarise K_I and IC_{50} data for both ketoconazole and cimetidine. Cimetidine K_I and IC_{50} values were determined, for the ECOD assay, in control microsomes and in microsomes from PB and β-NF pretreated rats (figures 5.2, 5.3 & 5.4). These values were determined for the MCOD assay in control and PB induced microsomes but not in β-NF induced microsomes since MCOD activity is not significantly different in such microsomes, from that observed in controls. Such values for the EROD assay were only determined in β-NF induced microsomes because of the low EROD activity in control and PB-induced rats. Rat pretreatment had no significant effect on the K_I values estimated for cimetidine using ECOD (0.4-0.6mM) or MCOD activities (0.2-0.4mM). Indeed these values for cimetidine inhibition were of the same order of magnitude irrespective of the assay or microsomal preparation.

Table 5.2 Inhibition of Microsomal Enzyme Activities by Cimetidine and Ketoconazole in Various Microsomal Preparations (K_i)

MICROSOMAL PREPARATION	KETOCONAZOLE K_i MEASUREMENTS (μM)		
	ECOD ASSAY	MCOD ASSAY	EROD ASSAY
Control	3.76 (1.79)	2.72 (1.20)	----------
	CIMETIDINE K_i MEASUREMENTS (mM)		
Control	0.56 (0.23)	0.24 (0.06)	----------
PB induction	0.41 (0.10)	0.42 (0.13)	----------
β-NF induction	0.36 (0.13)	----------	0.79 (0.22)

Values in parentheses are standard deviations.

Ketoconazole K_i values were determined for the ECOD and MCOD assays only in control microsomes (figure 5.1). K_i determined for the ECOD assay was not significantly different from that determined for MCOD. Comparing these values with those observed for the inhibitor cimetidine, indicated that ketoconazole was about 100 times more potent.

The concentration range used in the study was restricted by the limit of solubility of the compounds in DMF. Over the concentration range used the Dixon plot was observed to be biphasic for ketoconazole (0 - 50μM) with concentrations above 5μM giving a shallower slope. A similar trend was also observed with cimetidine but was not so apparent, possibly due to the concentration range used (0 - 5mM). This indicated that the inhibition was selective for particular isoenzymes of cytochrome P-450, a proportion being less affected by these inhibitors.

Table 5.3 Inhibition of Microsomal Enzyme Activities by Cimetidine and Ketoconazole in various Microsomal Preparations (IC_{50})

MICROSOMAL PREPARATION	KETOCONAZOLE IC_{50} MEASUREMENTS (μM)		
	ECOD ASSAY	MCOD ASSAY	EROD ASSAY
Control	4.4 (50) 3.7 (100) 9.8 (200)	2.5 (100) 3.0 (150) 4.6 (200)	-------- -------- --------
	CIMETIDINE IC_{50} MEASUREMENTS (mM)		
Control	0.77 (50) 0.80 (75) 0.77 (100) 1.20 (200)	0.34 (50) 0.34 (100) 0.48 (150) 0.87 (200)	-------- -------- -------- --------
PB induction	0.50 (5) 0.53 (25) 0.72 (50) 1.24 (200)	0.45 (25) 0.57 (50) 0.68 (100) 0.96 (200)	-------- -------- -------- --------
β-NF induction	0.45 (5) 0.66 (7.5) 1.45 (25) >5.0 (200)	-------- -------- -------- --------	0.59 (0.125) 0.79 (0.25) 1.37 (0.5) 2.15 (1.0)

Values in parentheses are substrate concentrations (μM)

Since IC_{50} values can be determined using one substrate concentration whereas K_I's require measurements at several concentrations, the former are frequently quoted. From classical enzyme kinetic equations for competitive inhibition in single enzyme systems a relationship between IC_{50}s and K_I can be derived, as shown in equation 5.1 (Dawes, 1969). This establishes the substrate concentration dependancy of IC_{50} values during competitive inhibition.

$$IC_{50} = K_I \cdot (1 + [S] / K_m) \qquad \text{equation 5.1}$$

This substrate dependency can clearly be seen in table 5.3. When the substrate concentration used was low, the IC_{50} value was comparable to the K_I, whereas at high concentrations, the IC_{50} value was much higher than the appropriate K_I. Since K_I values are independant of substrate concentration they can be more readily compared with other inhibition studies.

Figure 5.1 *In-vitro* Inhibition Data for Ketoconazole in Control Microsomes

A - % ECOD Activity *versus* log ketoconazole concentration

C - 1/ECOD Activity *versus* ketoconazole concentration

● - 50μM EC

■ - 100μM EC

◆ - 200μM EC

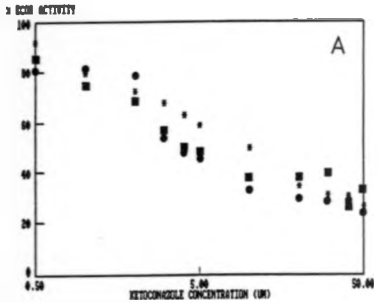
B - % MCOD Activity *versus* log ketoconazole concentration

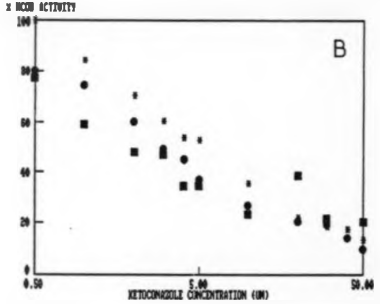
D - 1/MCOD Activity *versus* ketoconazole concentration

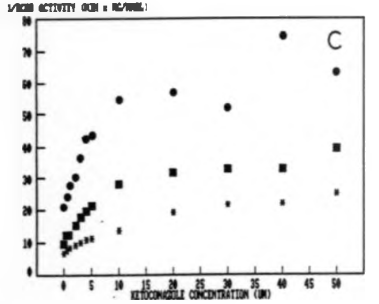
● - 100μM MC

■ - 150μM MC

◆ - 200μM MC


% HCDD ACTIVITY
A
KETOCONAZOLE CONCENTRATION (UM)


% HCDD ACTIVITY
B
KETOCONAZOLE CONCENTRATION (UM)


1/HCDD ACTIVITY (HCDD = NG/HOUR)
C
KETOCONAZOLE CONCENTRATION (UM)

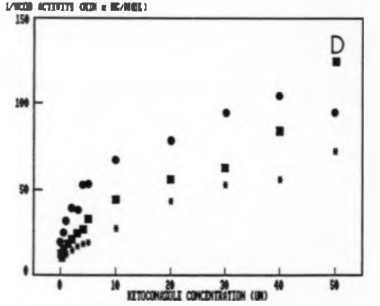

1/HCDD ACTIVITY (HCDD = NG/HOUR)
D
KETOCONAZOLE CONCENTRATION (UM)

Figure 5.2 *In-vitro* Inhibition Data for Cimetidine in Control Microsomes

A - % ECOD Activity *versus* log cimetidine concentration

C - 1/ECOD Activity *versus* cimetidine concentration

◆ - 50μM EC

✱ - 75μM EC

● - 100μM EC

■ - 200μM EC

B - % MCOD Activity *versus* log cimetidine concentration

D - 1/MCOD Activity *versus* cimetidine concentration

◆ - 50μM MC

✱ - 100μM MC

● - 150μM MC

■ - 200μM MC

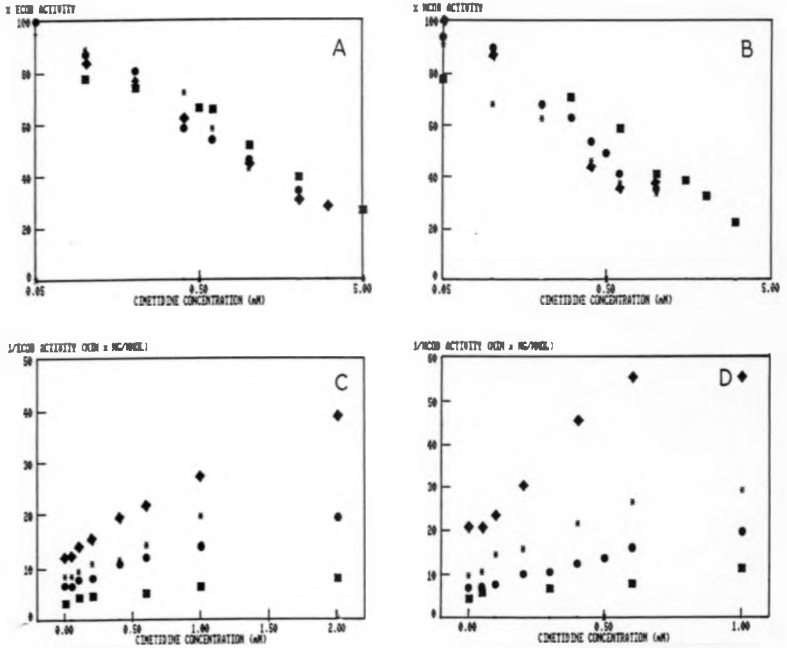

% ECH0 ACTIVITY
A
CIMETIDINE CONCENTRATION (mM)
% MCH0 ACTIVITY
B
CIMETIDINE CONCENTRATION (mM)
1/ECH0 ACTIVITY (MIN x NG/NMOL)
C
CIMETIDINE CONCENTRATION (mM)
1/MCH0 ACTIVITY (MIN x NG/NMOL)
D
CIMETIDINE CONCENTRATION (mM)

Figure 5.3 *In-vitro* Inhibition Data for Cimetidine in PB induced Microsomes

A - % ECOD Activity *versus* log cimetidine concentration

C - 1/ECOD Activity *versus* cimetidine concentration

◆ - 5μM EC

× - 25μM EC

■ - 50μM EC

● - 200μM EC

B - % MCOD Activity *versus* log cimetidine concentration

D - 1/MCOD Activity *versus* cimetidine concentration

◆ - 25μM MC

* - 50μM MC

● - 100μM MC

■ - 200μM MC

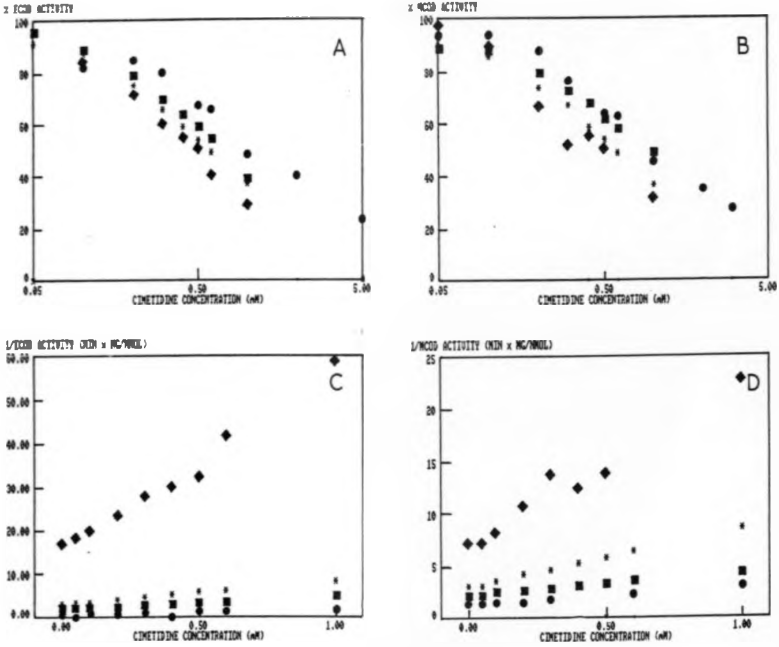

x ECCD ACTIVITY
A
CIMETIDINE CONCENTRATION (mM)
x MCCD ACTIVITY
B
CIMETIDINE CONCENTRATION (mM)
L/ECCD ACTIVITY (MIN x MG/ML)
C
CIMETIDINE CONCENTRATION (mM)
L/MCCD ACTIVITY (MIN x MG/ML)
D
CIMETIDINE CONCENTRATION (mM)

Figure 5.4 *In-vitro* Inhibition Data for Cimetidine in β-NF induced Microsomes

A - % ECOD Activity *versus* log cimetidine concentration

C - 1/ECOD Activity *versus* cimetidine concentration

◆ - 5μM EC

✱ - 7.5μM EC

● - 25μM EC

■ - 200μM EC

B - % EROD Activity *versus* log cimetidine concentration

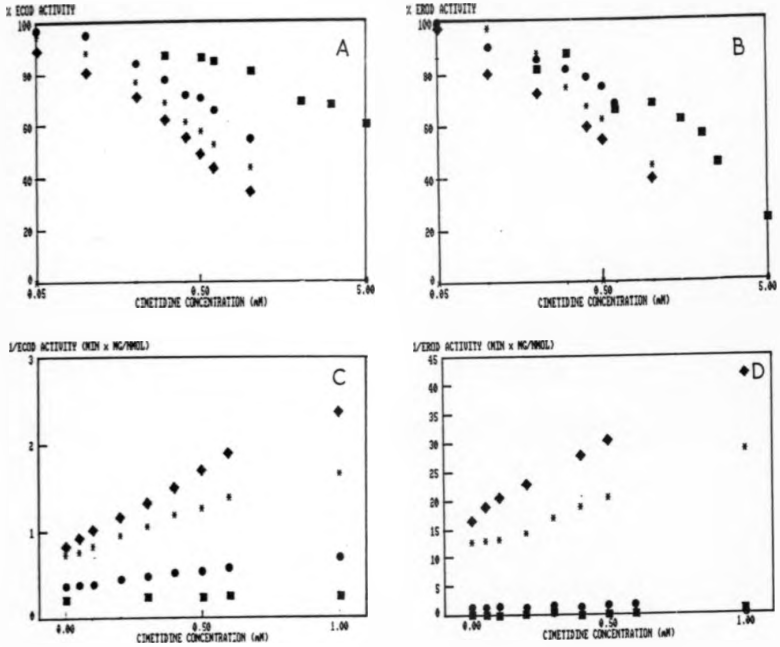
D - 1/EROD Activity *versus* cimetidine concentration

◆ - 0.125μM ER

✱ - 0.25 μM ER

● - 0.5 μM ER

■ - 1.0 μM ER


% ECOD ACTIVITY
A
100
80
60
40
20
0
0.05
0.50
5.00
CIMETIDINE CONCENTRATION (mM)
% ERCD ACTIVITY
B
100
80
60
40
20
0
0.05
0.50
5.00
CIMETIDINE CONCENTRATION (mM)
1/ECOD ACTIVITY (MIN x MG/NMOL)
C
3
2
1
0
0.00
0.50
1.00
CIMETIDINE CONCENTRATION (mM)
1/ERCD ACTIVITY (MIN x MG/NMOL)
D
45
40
35
30
25
20
15
10
5
0
0.00
0.50
1.00
CIMETIDINE CONCENTRATION (mM)

5.2.4 Conclusions

Spectral binding studies indicate both compounds to have two binding sites but that ketoconazole has a greater affinity than cimetidine for cytochrome P-450 (Reilly *et al.*, 1983; Lavrijsen *et al.*, 1987), with spectral binding constants for the high affinity site of 2.6μM and 0.7μM and the low affinity site of 104μM and 14μM for cimetidine and ketoconazole respectively.

In these studies ketoconazole was observed to be the more potent inhibitor, in control rat microsomes, of the *in-vitro* metabolism of 7-ethoxycoumarin and 7-methoxycoumarin. Dixon plots suggested that at certain inhibitor concentrations, interactions with ECOD and MCOD were competitive for both inhibitors. This was confirmed by the K_i and IC_{50} estimates being comparable at low substrate concentrations (equation 5.1). It is probably unwise, however, to apply such a type of classification, since the enzyme system contains a large number of different isoenzymes with different substrate/inhibitor specificities. Indeed the biphasicity of the plots confirms that the inhibition is selective towards particular isoenzymes of cytochrome P-450, and that others having a much lower affinity for the inhibitors.

Although IC_{50} estimations are much more quickly obtained than K_i values, it can be clearly seen that their value is limited. Not only do the values depend upon substrate concentration but they are also dependant on microsomal protein concentration (Gillette *et al.*, 1973). The studies presented here showed that IC_{50} values were only independant of substrate concentration at low substrate concentrations and that even lower substrate concentrations (ECOD assay) were required when microsomes from β-NF induced rats were used. This is indicative that the K_m of ECOD is lower in these microsomes than in controls, since IC_{50} only approximates to K_i when [S] is much lower than K_m. Unless the K_m of a reaction is known in order that a suitable substrate concentration may

be predicted, then K_i determination is of greater value. Generally drug metabolism studies where IC_{50} values have been determined have not appreciated the effect of substrate concentration and so have little value for comparison across studies.

5.3 *IN-VIVO* ASSESSMENT OF INHIBITION OF DRUG METABOLISM BY SINGLE DOSES OF KETOCONAZOLE IN THE RAT

5.3.1 Introduction

The previous study (section 5.2) confirmed the inhibitory potency of ketoconazole *in-vitro*. The present study was intended to examine whether such inhibition can be realized *in-vivo*. The effects of administering various single doses of ketoconazole (ip) on the kinetics of the model compound antipyrine was determined using a non-invasive breath test method (section 2.4.1).

5.3.2 Experimental Design

Male Sprague-Dawley rats (24) were divided into 8 groups (n=3), and given various doses of ketoconazole (ip 0, 1, 3, 5, 10, 15, 20 or 50 mg/Kg in cremophor: HCl 0.1N (1:9), 10ml/Kg). One hour later these rats received (N-methyl-^{14}C)-antipyrine (ip, 25mg/Kg, 1μCi/Kg, 2ml/Kg), were placed in all glass metabolism cages and $^{14}CO_2$ collected (section 2.4.1) over 7 h (controls) or 10 h (rats pretreated with ketoconazole).

5.3.3 Data Analysis

Samples (5.3.2) were processed as described in section 2.4.1. Antipyrine CER values were generated for each time point from averaged values for each three rat group (n=8) and plotted on semi-logarithmic paper. When inhibitors are acting under steady state conditions, K_i can be estimated from a derivation of the Michaelis-Menten equation (equation 5.2).

$$\frac{V}{C} = \frac{V_{max}}{K_m(1 + I/K_i)} \qquad \text{equation 5.2}$$

Where I is the steady state inhibitor concentration, V_{max} is the maximum rate of reaction, K_m is the Michaelis-Menten constant, C is the

substrate concentration, V the reaction rate and K_i is the inhibitor constant.

Using equation 5.2, an equation for clearance of antipyrine (CL) was derived (equation 5.3).

$$CL' = \frac{CL^{=}}{(1 + I^{=}/K_i)} \qquad \text{equation 5.3}$$

where $I^{=}$ is the inhibitor dose

' value in the presence of inhibitor

= control value

Similarly equations were derived for CER parameters:-

$$CER_{max}' = \frac{CER_{max}^{=}}{(1 + I^{=}/K_i)} \qquad \text{equation 5.4}$$

$$t_{1/2abs}' = t_{1/2abs}^{=} + I^{=}.\frac{t_{1/2abs}^{=}}{K_i} \qquad \text{equation 5.5}$$

Equation 5.4 was rearranged into a form which can be portrayed graphically (equation 5.6).

$$\frac{1}{CER_{max}'} = \frac{1}{CER_{max}^{=}} + \left(\frac{1}{CER_{max}^{=}} . \frac{I^{=}}{K_i}\right) \qquad \text{equation 5.6}$$

Application of equations 5.5 and 5.6 to the CER_{max} and $t_{1/2abs}$ data can therefore be used to determine values for K_i.

The CER data was also entered into the main frame computer and analysed by non-linear regression (NONLIN) using the equations below

(5.7 to 5.11). This enabled estimation of various kinetic parameters for the elimination of both antipyrine and ketoconazole and gave an estimate of the inhibitor dissociation constant (K_I). The method did not assume steady state levels of inhibitor, since it allowed for elimination during the experiment.

$$\frac{dD}{dt} = -k' \cdot D \qquad \text{equation 5.7}$$

(elimination of drug)

$$\frac{dN}{dt} = (f_m \cdot k' \cdot D) - k_m \cdot N \qquad \text{equation 5.8}$$

(formation of N) (elimination of N)

where :-

$$CER = k_e \cdot N \qquad \text{equation 5.9}$$

and

$$k' = \frac{k}{1 + I/K_I} \qquad \text{equation 5.10}$$

and

$$\frac{dI}{dt} = -k_x \cdot I \qquad \text{equation 5.11}$$

D - amount of drug in body
k' - elimination rate constant of drug in the presence of inhibitor (P1)
N - amount of metabolite formed
f_m - fraction of drug converted to norantipyrine
k_m - elimination rate constant of metabolite (P2)
CER- CO_2 exhalation rate
k - elimination rate constant of drug (control)
I - amount of inhibitor in body
K_I - inhibitor constant (P5)
k_x - inhibitor elimination rate constant (P4)

5.3.4 Results and Discussion

Averaged CER data for six of the eight groups studied are illustrated as log CER-time curves in figure 5.5. Inhibition of antipyrine elimination, observed even at the lowest dose of ketoconazole (1mg/Kg), was pronounced and dose-dependant. A progressive decrease in CER_{max} and AUC and notable dose-dependant changes in the shapes of the CER-time curves were observed. At low ketoconazole doses the peak was sharp but as the dose increased the peak broadened and occurred later. At the highest two doses the peak was replaced by a plateau region and the time to peak (t_{max}) had almost returned to the control value. At all doses other than 50mg/Kg, a post-peak curvilinear decline was observed in the CER-time profile. This may be explained by elimination of ketoconazole during the time course of the study. These terminal decline phases were nearly parallel.

Changes observed in the parameters CER_{max}, t_{max} and AUC are depicted in table 5.4. The 50mg/Kg dose decreased CER_{max} by 90% whereas the 10mg/Kg dose inhibited this parameter by 50%. The variation in t_{max} was unusual in that values increased up to a dose of 15mg/Kg and then gradually declined. The $AUC_{(0-\infty)}$, obtained from the cumulative CER, decreased with dose. However the AUC (table 5.6; P3) estimated by NONLIN was the same for each dose of inhibitor administered. This parameter gives an indication of the proportion of the antipyrine dose which is N-demethylated. The data therefore suggest that the fraction of drug undergoing N-demethylation was unaffected by ketoconazole administration.

Cumulative CER-time curves are illustrated in figure 5.6. Three t_{MRT}s were calculated from these cumulatives, using each individual dose $AUC_{(0-\infty)}$, the AUC_{∞} for the controls and each individual dose AUC_{∞} (Table 5.5). Although t_{MRT}s (own value 0-10 h) increased in a curvilinear fashion, the two t_{MRT}s increased linearly with

Figure 5.5 Typical (N-methyl-^{14}C)-antipyrine CER-Time Curves following administration of various Doses of Ketoconazole

A:-

■ - 1mg/Kg

- 5mg/Kg

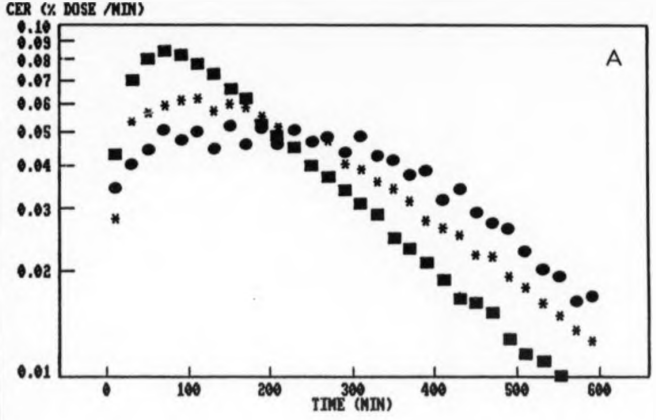
● - 10mg/Kg

B:-

● - 0mg/Kg

■ - 20mg/Kg

- 50mg/Kg


CER (% DOSE /MIN)
0.10
0.09
0.08
0.07
0.06
0.05
0.04
0.03
0.02
0.01
A
0
100
200
300
400
500
600
TIME (MIN)

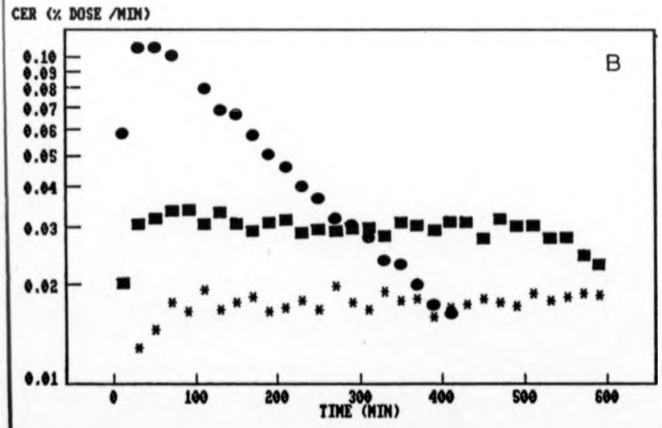

CER (% DOSE /MIN)
0.10
0.09
0.08
0.07
0.06
0.05
0.04
0.03
0.02
0.01
B
0
100
200
300
400
500
600
TIME (MIN)

Table 5.4 Effects of various Doses of Ketoconazole on Maximum $^{14}CO_2$ Exhalation Rate (CER_{max}), t_{max} and $AUC_{0-10\ h}$ following (N-methyl-^{14}C)-antipyrine Administration

KETOCONAZOLE DOSE (mg/Kg)	CER_{max} (% dose/min)	t_{max} (min)	$AUC_{0-10\ h}$ (%)
0	0.103 (0.007)	50 (10)	22.6 (1.0)
1	0.084 (0.005)	70 (10)	23.0 (1.3)
3	0.079 (0.002)	90 (5)	25.1 (0.2)
5	0.060 (0.006)	90 (10)	22.8 (1.1)
10	0.051 (0.010)	130 (0)	22.9 (3.6)
15	0.042 (0.008)	210 (20)	19.3 (1.5)
20	0.032 (0.002)	150 (10)	17.7 (0.2)
50	0.018 (0.001)	130 (5)	10.3 (0.9)

increasing dose (figure 5.8). This is explained by the fact that, at the three highest doses of ketoconazole, the % of dose N-demethylated during the time course of the experiment was much less than that for the controls and thus the time to 50%AUC (0-10h) was reduced. Linear relationships were observed when $t_{max\ dose}$ and $1/CER_{max}$ were plotted against dose of ketoconazole administered (figure 5.7 and 5.8). Estimates of K_I were determined from these graphs (table 5.7) using the equations 5.5 and 5.6 respectively.

The presence of terminal curvilinear decline phases in most of the CER-time profiles (figure 5.5) indicated that ketoconazole has a short half-life relative to that of antipyrine. Application of steady state inhibition kinetics (equations 5.6 & 5.7) was therefore thought to be inappropriate. Literature values indicate the half-life of ketoconazole to be around 1-2 h (Gascoigne *et al.*, 1981). It was thought appropriate therefore that the steady state inhibition equation be modified to

Table 5.5 Effects of various doses of ketoconazole on $t_{AUC_{0\infty}}$ following (N-methyl-^{14}C)-antipyrine Administration

KETOCONAZOLE DOSE (mg/Kg)	ANTIPYRINE PARAMETERS		
	TIME TO REACH 50% OF AUC (min)		
	A1	A2	A3
0	120.0	136.7	136.7
1	152.0	166.8	163.0
3	160.5	158.6	159.1
5	199.3	218.3	216.6
10	238.7	259.2	262.0
15	263.3	332.3	317.3
20	284.2	400.8	400.6
50	404.0	709.5	681.3

A1 - $t_{AUC_{0\infty}}$ using the averaged AUC_{0-t_n} obtained for each dose
A2 - $t_{AUC_{0\infty}}$ using the averaged $AUC_{0\infty}$ obtained from controls
A3 - $t_{AUC_{0\infty}}$ using the averaged $AUC_{0\infty}$ obtained for each dose

incorporate a term for inhibitor elimination (equations 5.7 to 5.11). This was applied to the CER data using a non-linear regression program (NONLIN). The non-linear regression estimates for the kinetic parameters of antipyrine and ketoconazole are summarised in table 5.6. The estimates for the initial accrual rate constant (k_a) and terminal elimination rate constant (k') of antipyrine CER, were highly predictive at (56.13 ± 2.13) x 10^{-3} and (5.43 ± 0.05) x 10^{-3} min^{-1} respectively. These are equivalent to an initial accrual half-life of 12 min and a terminal half-life of 128 min for antipyrine. Similarly the data predicted that the fraction of the antipyrine dose undergoing N-demethylation in controls to be 24.5 ± 0.5 % which is close to the

Figure 5.6 The Effects of various Ketoconazole Doses upon Cumulative $^{14}CO_2$ exhaled following (N-methyl-^{14}C)-antipyrine Administration

● - cremophor diluent controls

✱ - Ketoconazole 10mg/Kg

■ - Ketoconazole 20mg/Kg

● - Ketoconazole 50mg/Kg

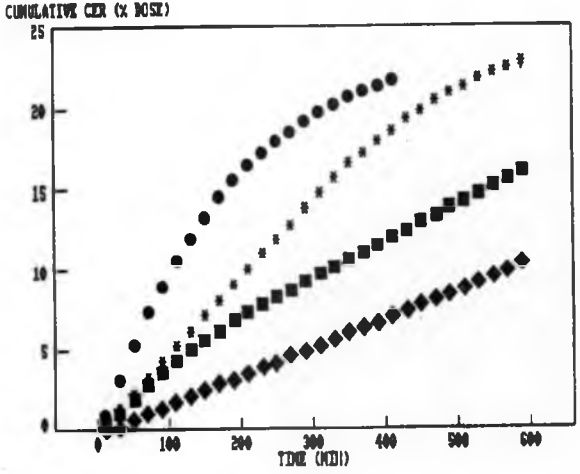
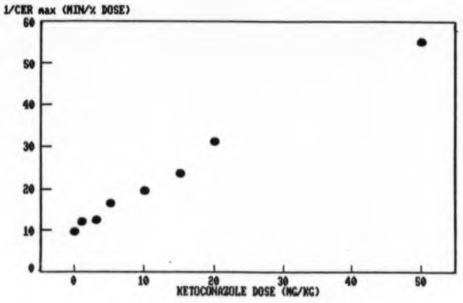

CUMULATIVE CER (x BOSE)
25
20
15
10
5
0
0
100
200
300
400
500
600
TIME (MIN)

Figure 5.7 Relationship Between Reciprocal $^{14}CHR_{max}$ following (N-methyl-^{14}C)-antipyrine Administration and Ketoconazole Dose

Figure 5.8 The Effects of various Ketoconazole Doses upon Determinations of the time to 50% AUC ($t_{AUC\ 50\%}$)

● - $t_{AUC\ 50\%}$ using each individual AUC.


1/CER max (MIN/% DOSE)
60
50
40
30
20
10
0
0
10
20
30
40
50
KETOCONAZOLE DOSE (MG/KG)

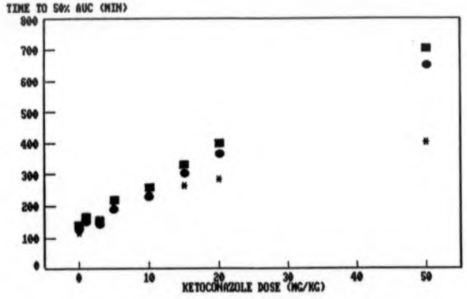

TIME TO 50% AUC (MIN)
800
700
600
500
400
300
200
100
0
0
10
20
30
40
50
KETOCONAZOLE DOSE (MG/KG)

Table 5.6 Estimates of Ketoconazole and Antipyrine Parameters obtained by Non-Linear Regression Analysis

DOSE	PARAMETERS				
(mg/Kg)	P1 (min^{-1}) x 10^{-2}	P2 (min^{-1}) x 10^{-2}	P3	P4 (min^{-1}) x 10^{-2}	P5 (mg/Kg)
1	5.46 (0.08)	54.10 (2.45)	0.243 (0.001)	3.22 (1.09)	3.32 (0.42)
3	5.41 (0.10)	56.63 (3.51)	0.249 (0.002)	17.53 (3.75)	2.81 (0.61)
5	5.48 (0.09)	53.99 (2.46)	0.246 (0.001)	5.42 (0.43)	4.03 (0.20)
10	5.50 (0.14)	56.78 (4.35)	0.251 (0.002)	5.57 (0.43)	4.40 (0.25)
15	5.36 (0.11)	55.89 (3.49)	0.237 (0.002)	5.16 (0.28)	5.03 (0.20)
20	5.38 (0.16)	60.27 (5.46)	0.248 (0.004)	2.55 (0.22)	5.71 (0.31)
50	5.44 (0.08)	55.28 (2.51)	0.243 (0.002)	1.29 (0.10)	7.06 (0.26)
Mean	5.43	56.13	0.245	----	4.62

P1 - accrual rate constant

P2 - elimination rate constant

P3 - fraction of drug N-demethylated

P4 - inhibitor elimination rate constant

P5 - inhibition constant

expected value. The predictions for the ketoconazole elimination rate (k_e) were more variable (5.82 x 10^{-3} min^{-1}) and gave a mean half-life for ketoconazole of 119min. The 5, 10 and 15 mg/Kg doses produced similar values for ketoconazole half-life (120 min) but the half-life increased following administration at the 20 and 50 mg/Kg dose levels. It is feasible that dose-dependent kinetics operate at the latter two doses resulting in a longer half-life estimate. Since ketoconazole was administered ip, however, saturation of absorption mechanisms may also be responsible.

Values of the K_I derived from this analysis from the 5, 10 and 15 mg/Kg doses were again more consistent (4.5 ± 0.5 mg/Kg) than those obtained by averaging all the values generated (4.6 ± 1.4 mg/Kg). Table 5.7 gives the estimates of K_I generated from the non-linear regression analysis (equations 5.7 to 5.11) and two graphical methods (Figures 5.7, and 5.8).

Estimates of K_I were in the range 4.6 to 13.8 mg/Kg for the NONLIN estimate and taucmax methods respectively. Since the graphical methods were based on steady state inhibition kinetics it was not surprising that these predicted ketoconazole to be a weaker inhibitor than the non-linear method which takes the elimination of the inhibitor into account.

Table 5.7 Estimates of K_I Made from the *In-vivo* Data

METHOD	K_I (mg/Kg)
A. Non-linear regression on CER-time profile	4.6
B. Graphically using CERmax	11.9
C. Graphically using taucmax (using A3)	13.8

5.3.5 Conclusions

As an antifungal agent, ketoconazole is administered at a dose of about 3 mg/Kg. Its use for prevention of infections in immunocompromised patients and in the treatment of prostatic cancer require much higher doses (20mg/Kg). In the present study, despite the rapid elimination ($t_{1/2}$ - 120 min), considerable inhibition of antipyrine elimination occurred at low doses of ketoconazole. Indeed the NONLIN estimate of K_I (4.6 mg/Kg), predicts considerable inhibition of metabolism at the dose levels used for the treatment of prostatic cancer.

Previous workers (Shaw and Houston, 1987) have simulated the effects of K_I and k_{in} of an inhibitor on drug and metabolite profiles. Two types of inhibition were considered, non-selective inhibition of all pathways of metabolism and the selective inhibition of one. The CER-time profiles generated here were consistent with the simulations generated for non-selective inhibition. In order to confirm this, urine metabolite analysis was required. Urine collected in the present study would have enabled detection of differences in urinary excretion of antipyrine metabolites but not formation clearances because of the elimination of ketoconazole during the studies. It was therefore decided to examine steady state ketoconazole inhibition of antipyrine clearance and individual metabolite formation clearances.

5.4 PHARMACOKINETICS OF KETOCONAZOLE MEASURED BY HPLC

5.4.1 Introduction

Although the pharmacokinetics of orally administered ketoconazole are well documented in humans, information on the kinetics in rats is sparse (Gascoigne *et al.*, 1981). Human studies have suggested that ketoconazole has dose-dependant kinetics (Huang *et al.*, 1986). These studies were undertaken in order to assess ketoconazole pharmacokinetics at different doses and to determine a suitable regimen for the steady state studies.

The methods of assay, for ketoconazole most frequently used have been microbiological (Clayton and Wingfield, 1981; Harvey *et al.*, 1980; Drouhet and Dupont, 1980). Such assays lack sensitivity and precision since they are indirect measurements of antifungal activity. Pharmacokinetic determinations with such assays have been unable to detect the terminal phase recently observed in studies by Huang *et al.* (1986). HPLC assays, which have greater sensitivity, have been developed subsequently (Alton, 1980; Andrews *et al.*, 1981; Badcock, 1984; Pascucci *et al.*, 1983; Riley and James, 1986; Swezey *et al.*, 1982). Whereas these methods require fairly large volumes of plasma sufficient sensitivity was attained for these preliminary studies using a modification of the method of Riley and James (1986).

5.4.2 Experimental Design

Male Sprague-Dawley rats were cannulated (section 2.4.2) and 12 h later placed in restraining cages. Various doses of ketoconazole (2.5mg/Kg n=7, 5mg/Kg n=8, 10mg/kg n=7) were administered in PEG:PPG (9:1) (1ml/Kg) *via* the jugular vein. Serial blood samples were taken from the carotid artery (n=9, 250µl) over 120 min (2.5mg/Kg), 150 min (5mg/kg) or 180 min (10mg/Kg). Samples were analysed as described in section 2.5.4.

5.4.3 Results and Discussion

The results of the plasma kinetic studies are shown in table 5.8 and figures 5.9 and 5.10. The dose range studied was within the range of doses used therapeutically for the treatment of fungal infections in man. There was a decrease in clearance of ketoconazole as the dose was increased from 2.5 to 10 mg/Kg with a consequent increase in half-life. When normalized for dose, the area under the plasma concentration-time profile (AUC) showed an increase as the dose of ketoconazole was increased (figure 5.10). The apparent volume of distribution was not, however, significantly altered (t-test) by changes in the administered dose. These results are consistent with previous reports in man (Daneshmand *et al.*, 1983; Huang *et al.*, 1986) of the dose-dependent kinetics of ketoconazole as indicated by the disproportionate increase in AUC with dose. The half-life and AUC observed, at the 10mg/Kg dose level, was consistent with the literature (Gamcoigne *et al.*, 1981) (table 5.8). Ketoconazole half-life following iv administration was much shorter than that determined by MOHLIN in the previous study for ip administration at corresponding dose levels. This discrepancy may have resulted from rate limiting absorption from the peritoneal cavity, due to the slow uptake from the oily vehicle in which the ketoconazole was administered in the previous study.

5.4.4 Conclusions

The purpose of these investigations was to obtain estimates of clearance and apparent volume of distribution for ketoconazole. On the basis of the pharmacokinetic parameters derived here, further studies were designed to produce a range of steady-state ketoconazole concentrations in rats which allowed study of the inhibitory interaction between ketoconazole and antipyrine. Although dose-dependent kinetics were observed, it was expected that fairly low steady state concentrations

would produce significant inhibition as judged by the previous study (section 5.3).

Table 5.8 Pharmacokinetic Parameters of Ketoconazole following Administration of various Doses

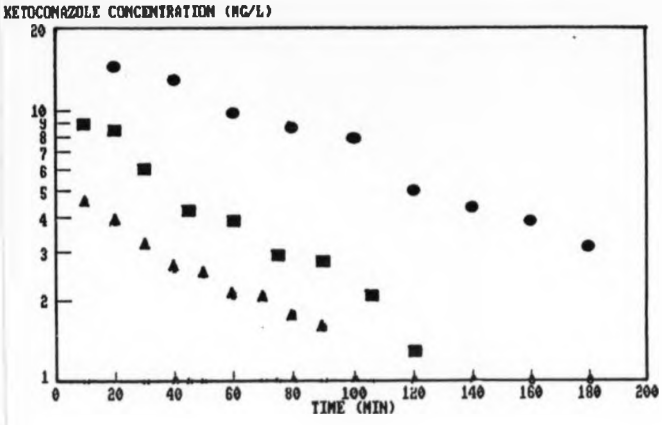
DOSE (mg/Kg)	n	CLEARANCE (ml/min/SRW)	HALF-LIFE (min)	VOLUME OF DISTRIBUTION (L/SRW)	AREA UNDER CURVE (mg/L x min)
2.5	7	2.09 (0.61)	49.1 (11.3)	0.14 (0.02)	343.7 (113.3)
5.0	8	1.94 (0.46)	41.8 (13.8)	0.11 (0.05)	*669.5 (189.1)
10.0	7	**1.35 (0.24)	**69.1 (13.2)	0.13 (0.03)	*1878.3 (410.3)

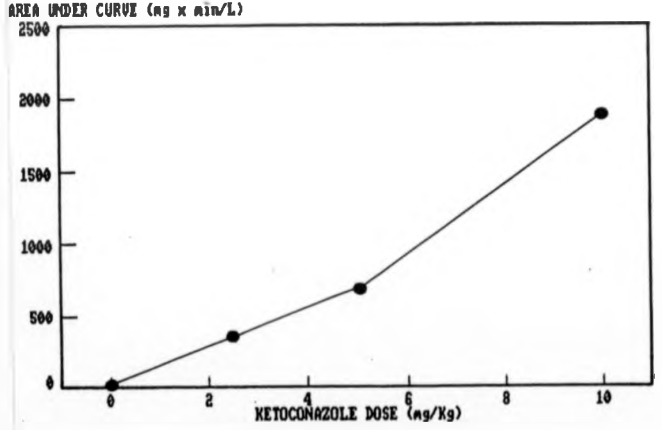
Significantly different from the 2.5mg/Kg dose;
* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$

Figure 5.9 Ketoconazole Plasma Concentration-Time Curves at Various Dosage Levels (Mean Data)

▲ - Ketoconazole 2.5mg/Kg
■ - Ketoconazole 5.0mg/Kg
● - Ketoconazole 10.0mg/Kg

Figure 5.10 Relationship Between the AUC and Ketoconazole Dose Administered


KETOCONAZOLE CONCENTRATION (NG/L)
20
10
9
8
7
6
5
4
3
2
1
0
20
40
60
80
100
120
140
160
180
200
TIME (MIN)


AREA UNDER CURVE (ng x min/L)
2500
2000
1500
1000
500
0
0
2
4
6
8
10
KETOCONAZOLE DOSE (mg/Kg)

5.5 INHIBITION OF ANTIPYRINE BY STEADY STATE CONCENTRATIONS OF KETOCONAZOLE

5.5.1 Introduction

The previous *in-vivo* inhibition study (section 5.3) suggested that ketoconazole was a fairly potent inhibitor of antipyrine metabolism. The studies only produced qualitative information, however, since the kinetics of ketoconazole were not monitored. Other problems arose due to the rapid elimination of ketoconazole during the time course of the experiment. It was therefore decided to design a steady state inhibition study by infusing ketoconazole throughout the experiment and simultaneously monitoring ketoconazole plasma concentrations and antipyrine kinetics following administration of a bolus dose. A suitable regimen for ketoconazole was predicted using the pharmacokinetic parameters previously determined (section 5.4).

5.5.2 Experimental Design

Male Sprague-Dawley rats were cannulated (section 2.4.2) and 12 h later were placed in restraining cages. Various bolus doses of ketoconazole (0 to 2.6 mg/Kg, 1ml/Kg) were administered followed by a bolus dose of (N-methyl-^{14}C)-antipyrine (50mg/Kg, 1ml/Kg, 1μCi/Kg). Ketoconazole infusions were then commenced (0-2.5 mg/h/Kg, 0.15 ml/h in PEG:PPG 9:1). Serial blood samples were taken from the carotid artery (n=9, 150μl-antipyrine; n=5, 250μl-ketoconazole) over 10 h. Samples were analysed as described in section 2.5.1 for antipyrine and section 2.5.4 for ketoconazole.

At the end of the experiment, rats were killed with a lethal dose of pentobarbitone (2ml/Kg) and their bladders drained. Urine samples were collected and treated as described in sections 2.4.1. Urine was analysed for antipyrine metabolites (section 2.5.5).

Radio-labelled antipyrine was used in order to estimate the recovery of administered antipyrine, essential since urine was only collected for 10 h.

5.5.3 Data Analysis

Suitable dosage regimen were determined using the equations 5.12 and 5.13 for bolus dose and infusion rate respectively.

$$\text{Bolus dose} = C_{ss} \cdot V_d \qquad \text{equation 5.12}$$

$$\text{Infusion rate} = C_{ss} \cdot CL \qquad \text{equation 5.13}$$

C_{ss} - steady state concentration

V_d - apparent volume of distribution

CL - clearance

Equation 5.13 can be rearranged into a form which allows determination of ketoconazole intrinsic clearance, V_{max} and K_m for compounds exhibiting Michaelis-Menten kinetics:

$$\frac{C_{ss}}{R} = \frac{1}{CL} = \frac{K_m + C_{ss}}{V_{max}} = \frac{K_m}{V_{max}} + \frac{C_{ss}}{V_{max}} \qquad \text{equation 5.14}$$

R - infusion rate

K_m - Michaelis-Menten constant

V_{max} - maximum velocity of the reaction

Two inhibition models were fitted using a nonlinear regression program (PHARM 6) to the clearance data derived for antipyrine and its metabolites (equations 5.15 and 5.16). Model 1 is a single binding site

model, whilst model II includes a constant term which allows for the presence of ketoconazole-resistant isoenzymes involved in the metabolism of antipyrine. These models have previously been fitted to similar data for cimetidine (Adedoyin *et al.*, 1987).

Model I:-

$$CL_i = \frac{CL}{1 + C_{ss}/K_i} \qquad \text{equation 5.15}$$

CL_i - total or formation clearance in the presence of inhibitor

CL - clearance in the absence of inhibition

K_i - inhibitor constant

Model II:-

$$CL_i = \frac{CL_1}{1 + C_{ss}/K_i} + CL_2 \qquad \text{equation 5.16}$$

CL_1 - clearance for enzymes susceptible to ketoconazole inhibition

CL_2 - clearance for enzymes resistant to ketoconazole inhibition

5.5.4 Results and Discussion

The dose dependency of ketoconazole pharmacokinetics observed in section 5.4 were confirmed by these steady-state studies (figure 5.11, table 5.9). At concentrations above 5mg/L, the steady-state concentrations achieved were greater than those predicted by the single dose (2.5mg/Kg) study. Linearisation of the relationship between infusion rate and steady-state concentration (equation 5.14 and figure 5.12) produced an estimate of ketoconazole intrinsic clearance of 2.9 ml/min/SRV. Since

the preliminary studies produced a clearance estimate of 2.1 ml/min/SRV at a dose of 2.5mg/Kg, this suggested that dose-dependency was evident even at this dose level. Values determined for V_{max} and K_m were 0.8mg/h/SRV and 4.5mg/L respectively. The ketoconazole concentration-time profile (figure 5.9) for the 2.5mg/Kg had a C_{ss} of 4.8mg/L and so concentrations largely fell close to the K_m value of 4.5mg/L. This suggested that only at doses below 2.5mg/Kg would the kinetics of ketoconazole be linear and higher doses will show dose-dependent kinetics. The infusion data and the previous single dose data are thus consistent (section 5.4).

The data for antipyrine are shown in figure 5.13 and table 5.10. There was a decrease in total plasma clearance of antipyrine as the steady-state concentration of ketoconazole increased with a consequent increase in half-life. The apparent volume of distribution of antipyrine was not significantly altered (one way ANOVA) by ketoconazole administration. At maximal ketoconazole inhibition, antipyrine clearance was only decreased by 55%. The formation clearances of all the metabolites of antipyrine were decreased by ketoconazole (table 5.11) to a similar extent. The NOR pathway was inhibited by 60% whilst the 3H and 4H pathways were inhibited by about 50% (table 5.11 and figure 5.14).

Metabolite urinary excretion data was expressed as a proportion of antipyrine eliminated during the experiment. Although the amount excreted in 10 h was only a fraction of the total, it was considered more appropriate to analyse urine excreted under steady state ketoconazole concentration conditions; relative values for the metabolites being similar to that obtained for complete collection. Although the fractions of 3-hydroxymethylantipyrine and 4-hydroxyantipyrine excreted were essentially unaffected by ketoconazole inhibition, there was a significant decrease in f_m for norantipyrine at all of the mean steady state concentrations except the highest. A marked

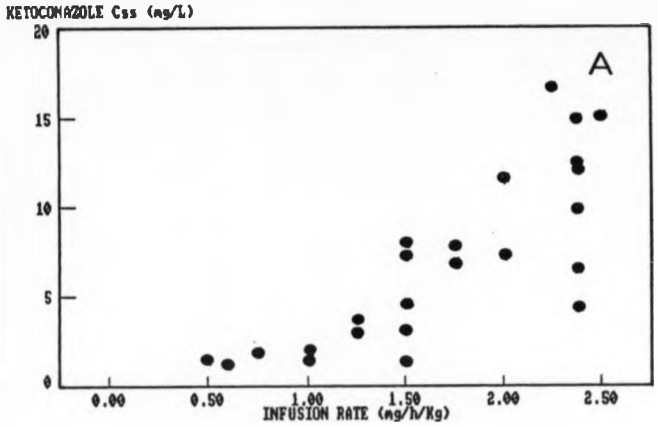
increase in renal excretion was also apparent (table 5.13 and figure 5.15).

Antipyrine clearance and renal clearance data together with the antipyrine metabolite formation clearance data were analysed using two models (equation 5.15 and 5.16; table 5.14). Model I produced a good estimate of antipyrine clearance in control animals, but predicted much lower clearances than were observed at high ketoconazole steady state concentrations. This was because this model assumed that ketoconazole could completely inhibit antipyrine metabolism at a particular concentration. In fact following a rapid decrease in clearance at low ketoconazole steady-state concentrations, there appeared to be very little further alteration in antipyrine clearance at ketoconazole concentrations above 5mg/L. This suggested that a proportion of the isoenzymes involved in the metabolism of antipyrine were extremely sensitive to ketoconazole inhibition, whilst others were resistant. Similar trends were observed in the metabolite data (figure 5.16). Model II, which allows for classes of resistant and sensitive isoenzymes clearly produced a better fit with the data (figures 5.16 to 5.19). Indeed the goodness of fit criteria (table 5.15) for all the clearance terms measured were consistently more negative for model II indicating that this was more appropriate than model I. The clearance parameters estimated from the fitting of model II were in keeping with the data (table 5.12). Model II produced two clearance terms, one of which indicated the contribution to antipyrine clearance made by ketoconazole-sensitive (CL_1) and -insensitive (CL_2) isozymes. Antipyrine renal clearance was unaffected by ketoconazole inhibition (figure 5.20).

Estimates for ketoconazole K_i were 0.9mg/L (1.7μM) for the nor- and 4-hydroxyantipyrine metabolites and 1.2mg/L (2.2μM) for the 3-hydroxymethylantipyrine metabolite and antipyrine itself (table 5.14).

Figure 5.11 Relationship Between Ketoconazole Steady State Concentration and its Infusion Rate

Figure 5.12 Ketoconazole Steady State Concentration: Infusion Rate Ratio versus Ketoconazole Steady State Concentration


KETOCONAZOLE Css (mg/L)
20
15
10
5
0
A
0.00
0.50
1.00
1.50
2.00
2.50
INFUSION RATE (mg/h/Kg)

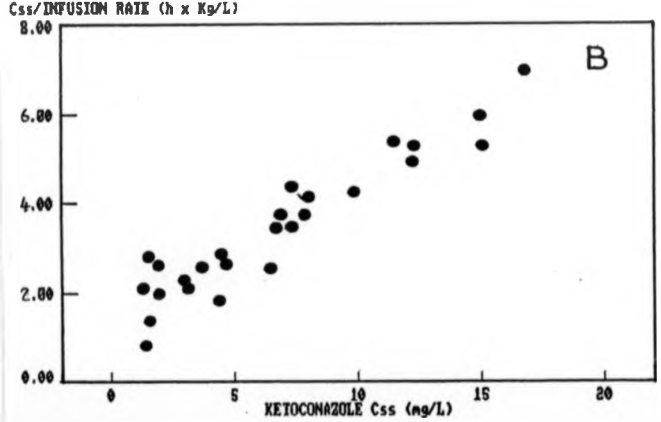

Css/INFUSION RATE (h x Kg/L)
8.00
6.00
4.00
2.00
0.00
B
0
5
10
15
20
KETOCONAZOLE Css (mg/L)

Table 5.9 Steady State Concentrations of Ketoconazole following Administration of various Doses

Nominal C_{ss} (mg/L)	Bolus Dose (mg/Kg)	Infusion Rate (mg/h/Kg)	Actual C_{ss} (mg/L)
0 (n=7)	1ml/Kg	0.15ml/h	0
1.5 (n=6)	0.92 (0.37)	0.90 (0.37)	1.49 (0.25)
3.0 (n=6)	1.61 (0.42)	1.57 (0.42)	3.84 (0.73)
3.5 (n=7)	1.85 (0.30)	1.81 (0.31)	7.16 (0.56)
4.0 (n=7)	2.38 (0.16)	2.33 (0.16)	13.19 (2.43)

Table 5.10 Pharmacokinetic Parameters of Antipyrine in the Presence of Various Steady State Concentrations of Ketoconazole

Ketoconazole C_{ss} (mg/L)	Clearance (ml/min/SRW)	Half-life (min)	Volume of Distribution (L/SRW)
0 (n=7)	2.07 (0.40)	100.0 (19.3)	0.30 (0.02)
1.49 (0.25) (n=6)	*1.36 (0.29)	*156.2 (31.7)	0.30 (0.03)
3.84 (0.73) (n=6)	*1.26 (0.23)	*175.3 (25.1)	0.31 (0.03)
7.16 (0.56) (n=7)	*0.96 (0.19)	*217.9 (35.6)	0.29 (0.03)
13.19 (2.43) (n=7)	*0.92 (0.18)	*236.1 (45.8)	0.31 (0.03)

Signicantly different from controls * $p < 0.001$
Determined using one way ANOVA

Figure 5.13 Typical Antipyrine Blood Concentration-Time Curves During Steady State Inhibition by Ketoconazole

▲ - Ketoconazole 0mg/L

◆ - Ketoconazole 1.3mg/L

■ - Ketoconazole 4.5mg/L

● - Ketoconazole 16.7mg/L

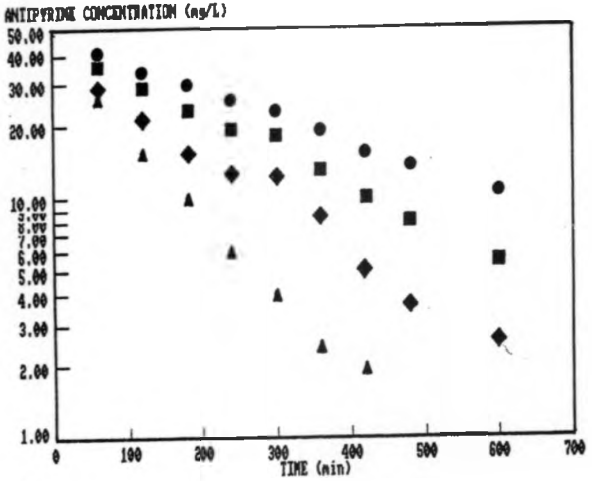

AMISULPRIDE CONCENTRATION (mg/L)
50.00
40.00
30.00
20.00
10.00
9.00
8.00
7.00
6.00
5.00
4.00
3.00
2.00
1.00
0
100
200
300
400
500
600
700
TIME (min)

Table 5.11 The Effects of Ketoconazole at various Steady State Concentrations on renal clearance of antipyrine and formation clearance of its metabolites

KETOCONAZOLE STEADY STATE CONCENTRATION (mg/L)	FORMATION CLEARANCE (ml/min/SRW)			RENAL CLEARANCE (ml/min/SRW)
	4OH-	NOR-	3OHMe-	
0 (n=7)	0.43 (0.09)	0.27 (0.05)	0.39 (0.11)	0.08 (0.03)
1.49 (0.25) (n=6)	*0.26 (0.12)	*0.15 (0.03)	*0.26 (0.12)	0.04 (0.02)
3.84 (0.73) (n=6)	*0.26 (0.13)	*0.14 (0.05)	*0.25 (0.10)	0.07 (0.02)
7.16 (0.56) (n=7)	*0.20 (0.06)	*0.09 (0.02)	*0.17 (0.04)	0.06 (0.02)
13.19 (2.43) (n=7)	*0.21 (0.07)	*0.11 (0.03)	*0.19 (0.07)	0.06 (0.01)

Significantly different from control;
* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$
Determined by one way ANOVA

Table 5.12 Parameters estimated from model 2

0 (n=7)	0.43	0.27	0.39	0.06
1.49 (0.25) (n=6)	0.28	0.16	0.25	0.06
3.84 (0.73) (n=6)	0.24	0.12	0.21	0.06
7.16 (0.56) (n=7)	0.22	0.11	0.19	0.06
13.19 (2.43) (n=7)	0.21	0.10	0.18	0.06

Table 5.13 The effect of various Ketoconazole Steady State Concentrations on urinary recovery of antipyrine and its Metabolites

KETOCONAZOLE STEADY STATE CONCENTRATION (mg/L)	% RECOVERED (FUNCTION OF DOSE METABOLIZED IN 10h)			
	4H-	NOR-	3OHMe-	AP
0 (n=7)	20.4 (4.6)	13.0 (1.9)	17.8 (4.1)	3.7 (2.1)
1.49 (0.25) (n=6)	17.8 (4.9)	11.3 (2.3)	18.2 (4.4)	3.1 (1.6)
3.84 (0.73) (n=6)	21.2 (7.6)	*10.5 (2.2)	19.0 (5.4)	*5.3 (1.4)
7.16 (0.56) (n=7)	20.8 (3.7)	**9.3 (1.4)	17.8 (3.0)	**6.1 (1.4)
13.19 (2.43) (n=7)	22.4 (5.7)	11.3 (2.8)	20.6 (4.4)	**7.0 (1.3)

Significantly different from control;
* $p < 0.05$; ** $p < 0.01$
Determined using one way ANOVA

Table 5.14 Parameter estimates for the two models (eq. 5.15 & 5.16) fitted to the antipyrine and antipyrine metabolite Data

PARAMETER	PARAMETER ESTIMATES				
	Model 1		Model 2		
	CL (ml/min/SRW)	K_1 (mg/L)	CL_1 (ml/min/SRW)	K_{11} (mg/L)	CL_2 (ml/min/SRW)
CL(AP)	1.97 (0.10)	6.96 (1.30)	1.34 (0.14)	1.19 (0.47)	0.81 (0.11)
CL(4H)	0.39 (0.03)	8.81 (2.82)	0.24 (0.05)	0.89 (0.83)	0.19 (0.04)
CL(3H)	0.36 (0.03)	8.41 (2.72)	0.22 (0.05)	1.19 (1.11)	0.17 (0.04)
CL(M)	0.26 (0.02)	4.19 (0.86)	0.19 (0.02)	0.93 (0.46)	0.08 (0.02)

Model 1 - equation 5.15; Model 2 - equation 5.16

Values in parentheses are standard errors from the mean (SE)

Table 5.15 Goodness of fit criteria for the two models (eq. 5.15 & 5.16) fitted to the antipyrine and antipyrine metabolite data

Metabolite	2 x Log Likelihood		Akaike		Schwarz	
	Model I	Model II	Model I	Model II	Model I	Model II
CL(AP)	13.49	- 1.99	4.75	- 4.00	3.19	- 6.33
CL(4H)	-61.02	-66.67	-32.51	-36.34	-34.01	-38.58
CL(3H)	-65.13	-68.51	-34.56	-37.26	-36.06	-39.50
CL(M)	-112.75	-124.24	-58.37	-65.12	-59.87	-67.37

Figure 5.14 Relationships Between Antipyrine Metabolite Formation Clearance and Renal Clearance and Ketoconazole Steady State Concentration

● - 4-hydroxyantipyrine

■ - 3-hydroxymethylantipyrine

✱ - norantipyrine

◆ - antipyrine renal clearance

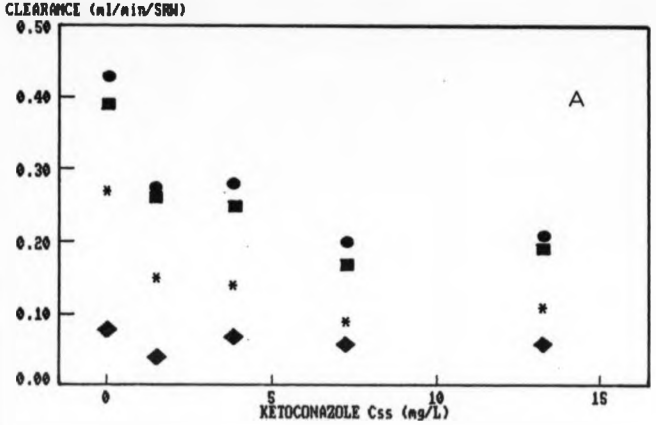
Figure 5.15 Relationships Between Antipyrine Metabolite Urinary Excretion and Renal Excretion and Ketoconazole Steady State Concentration

● - 4-hydroxyantipyrine

■ - 3-hydroxymethylantipyrine

✱ - norantipyrine

◆ - antipyrine renal clearance


CLEARANCE (ml/min/SRM)
0.50
0.40
0.30
0.20
0.10
0.00
A
0
5
10
15
KETOCONAZOLE Css (mg/L)

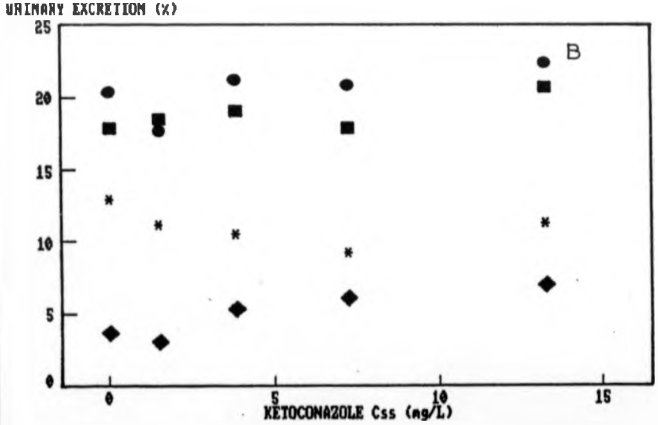

URINARY EXCRETION (%)
25
20
15
10
5
0
B
0
5
10
15
KETOCONAZOLE Css (mg/L)

Figure 5.16 Relationship between Antipyrine Clearance and ketoconazole Steady State Concentration

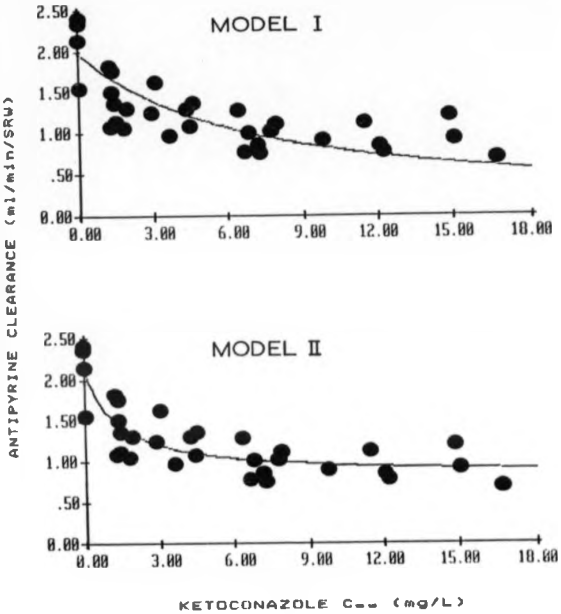

MODEL I
MODEL II
ANTIPYRINE CLEARANCE (ml/min/SRW)
KETOCONAZOLE Css (mg/L)
2.50
2.00
1.50
1.00
.50
0.00
0.00
3.00
6.00
9.00
12.00
15.00
18.00

Figure 5.17 Relationship between 4-hydroxyantipyrine Clearance and Ketoconazole Steady State Concentration

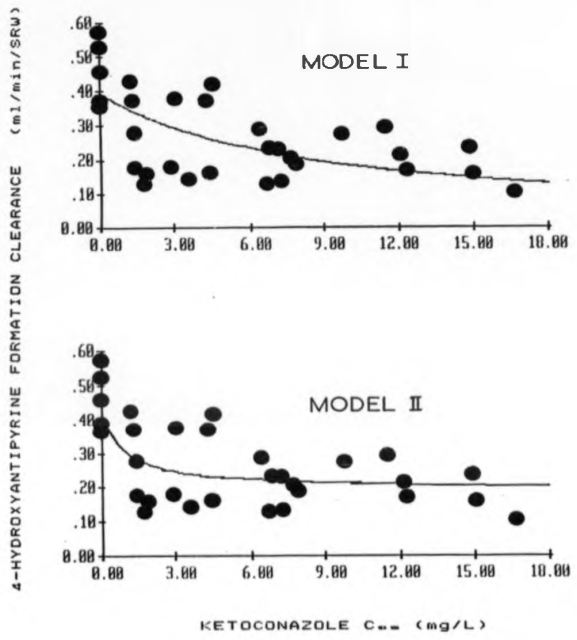

MODEL I
MODEL II
4-HYDROXYANTIPYRINE FORMATION CLEARANCE (ml/min/SRW)
KETOCONAZOLE Css (mg/L)
.60
.50
.40
.30
.20
.10
0.00
0.00
3.00
6.00
9.00
12.00
15.00
18.00

Figure 5.18 Relationship between Norantipyrine Clearance and Ketoconazole Steady State Concentration

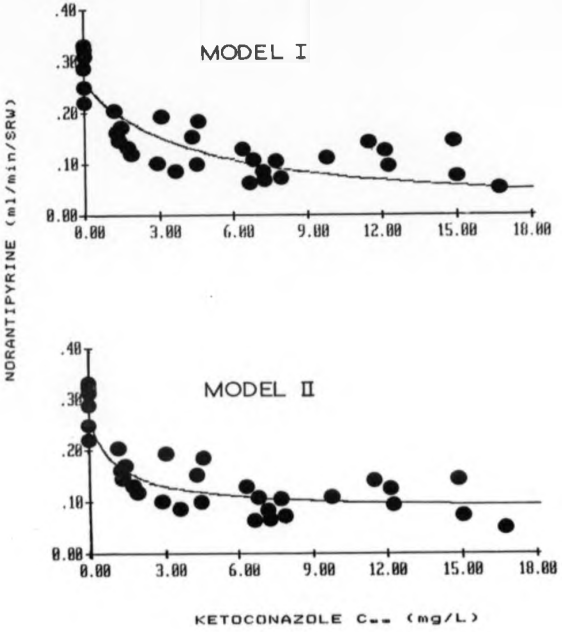

MODEL I
MODEL II
NORANTIPYRINE (ml/min/SRW)
.40
.30
.20
.10
0.00
0.00
3.00
6.00
9.00
12.00
15.00
18.00
KETOCONAZOLE C-- (mg/L)

Figure 5.19 Relationship between 3-hydroxymethylantipyrine Clearance and Ketoconazole Steady State Concentration

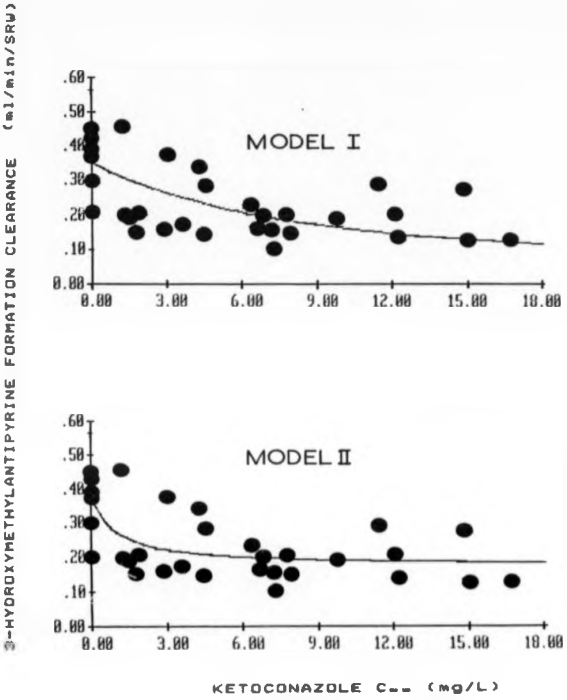
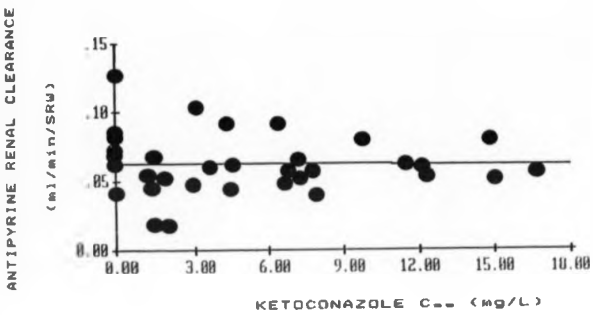

3-HYDROXYMETHYLANTIPYRINE FORMATION CLEARANCE (ml/min/SRW)
MODEL I
MODEL II
.60
.50
.40
.30
.20
.10
0.00
0.00
3.00
6.00
9.00
12.00
15.00
18.00
KETOCONAZOLE Css (mg/L)

Figure 5.20 Relationship between Antipyrine Renal Clearance and Ketoconazole Steady State Concentration


ANTIPYRINE RENAL CLEARANCE
(ml/min/SRW)
.15
.10
.05
0.00
0.00
3.00
6.00
9.00
12.00
15.00
18.00
KETOCONAZOLE C.. (mg/L)

5.5.5 Conclusions

This study has demonstrated that following acute administration, ketoconazole inhibits *in-vivo* metabolism in the rat and confirms the previous single dose studies in section 5.4 and in the studies by Meredith *et al.*, (1985). The inhibition of antipyrine elimination by ketoconazole results from the nonspecific inhibition of oxidative metabolism of antipyrine to its three main metabolites 3-hydroxymethylantipyrine, 4-hydroxyantipyrine and norantipyrine, as measured by their individual formation clearances. The increase in half-life is as a consequence of the decrease in clearance. The decrease in antipyrine clearance is dependent upon the plasma ketoconazole concentration.

These studies confirm the importance of formation clearance data determination since apart from an initial decrease in norantipyrine excretion, there was little difference between control and inhibited urinary metabolite excretion. An increase in the fraction of unchanged antipyrine in urine occurred and this probably arose as a result of the increased concentrations of antipyrine present in the plasma since there was no change in the renal clearance.

Similar studies with cimetidine (Adedoyin *et al.*, 1987) showed a marked contrast with the ketoconazole data. Cimetidine produced a selective effect on the antipyrine metabolite pathways, having greatest affinity for the isoenzymes of the 4-hydroxyantipyrine pathway (K_I 0.04mg/L = 0.2μM) intermediate affinity for the isoenzymes of the norantipyrine pathway (K_I 0.25mg/L = 0.9μM) and least affinity for the isoenzymes of the 3-hydroxymethylantipyrine pathway (K_I 2.2mg/L = 8.7μM). The maximum inhibition was, however, similar for each pathway, at about 90%. These data indicate cimetidine to be the more potent overall inhibitor of antipyrine metabolism, having a greater capacity to affect most of the isoenzymes involved in antipyrine metabolism. In

comparison to cimetidine, ketoconazole had greater affinity for the isoenzymes involved in the formation of 3-hydroxymethylantipyrine (K_i = 2.2μM), similar affinity for those of norantipyrine (K_i = 1.7μM) but less for those of 4-hydroxyantipyrine (K_i = 1.7μM).

There is comparability between the inhibitory action of ketoconazole in both *in-vitro* and *in-vivo* metabolism. K_i determinations were of the order of 2 to 4μM for the *in-vitro* studies and of the order of 2 to 2.5μM for the steady state *in-vivo* studies. The CER study produced a K_i of 4.6 mg/Kg which, when adjusted using the volume of distribution, determined in section 5.4, is equivalent to a ketoconazole concentration of 8.2 mg/L (16μM). This estimate was much larger than that determined in the steady state study as a consequence of applying model II (equation 5.15). Indeed this value (16μM) is comparable to the K_i value generated by applying model I (equation 5.14) to the steady-state data (15μM). Despite the use of different substrates for the *in-vitro* and *in-vivo* investigations, similar inhibitory potencies for ketoconazole were found.

CHAPTER 6

FINAL DISCUSSION

Various *in-vitro* and *in-vivo* methods of assessing drug metabolizing capacity have been examined by means of induction studies with the classical inducers, β-NF and PB, and a third type of inducing agent, DEHP, and of inhibition studies with ketoconazole. Relationships between *in-vitro* and *in-vivo* parameters, in these studies, have been compared and assessed.

The *in-vitro* and *in-vivo* markers used in the induction studies were selected on the basis of their specificity for particular isoenzymes of cytochrome P-450. Studies were designed carefully to minimise inter- and intra-individual differences in drug metabolizing ability. In particular, *in-vitro* and *in-vivo* components were carried out on the same rats and on the same day, using a number of *in-vivo* and *in-vitro* markers. A range of drug metabolizing abilities were achieved in these rats using various doses of the P450I inducer, β-NF and the P450IIB inducer, PB.

In order to compare the specificity of the *in-vivo* markers, it was considered expedient to administer them simultaneously. This necessitated preliminary studies to determine whether or not co-administration of the *in-vivo* markers would affect their individual pharmacokinetic parameters. Such studies indicated that at the doses administered, no effects were observed in determined pharmacokinetic parameters for either antipyrine, tolbutamide or theophylline when antipyrine was administered with either tolbutamide or theophylline to rats. These initial studies also identified suitable dose levels for each drug, particularly important for theophylline since at high doses, its kinetics are dose-dependent. It was determined that at the dose of theophylline chosen for these studies, theophylline was not exhibiting dose-dependent kinetics.

In-vivo and *in-vitro* dose response data generated during the correlation studies were utilised to predict doses of inducer required to achieve as wide a range as possible in drug metabolizing capacity. Although induction studies frequently utilise doses of PB of 80mg/Kg and β-NF of 100mg/Kg, these studies indicated that maximal induction of most parameters were achieved at a dose of about 30mg/Kg, hence the majority of rats received doses lower than 30mg/Kg.

The elimination of antipyrine and theophylline was induced by both PB and β-NF administration, however the latter was the more potent inducer. Tolbutamide elimination was only susceptible to induction by PB. Previous studies have indicated that use of antipyrine metabolite formation clearance data can in certain situations improve *in-vivo* correlations (Tennissen *et al.*, 1986; Van der Graaff *et al.*, 1983a). The data generated in these studies indicated that although CL(4H) correlated more strongly than CL(AP) with *in-vitro* parameters in β-NF induced rats, none of the metabolite formation clearances improved upon the correlations observed between the parent drug clearance (CL(AP)) and *in-vitro* markers in PB induced rats. The strong correlation between CL(TH) and CL(4H) was in keeping with their superiority as *in-vivo* markers of increased levels of P450I following β-NF induction. The *in-vivo* parameter which produced the strongest relationships with *in-vitro* parameters following PB induction was CL(TOL).

Correlations between *in-vitro* markers and the various *in-vivo* markers were consistently stronger for the more specific markers of the particular type of induction employed. For example EROD, the enzyme specific for P450I and MCOD the enzyme specific for P450IIB, only produced strong correlations with other markers in β-NF induced and PB induced rats respectively. In contrast, strong correlations were observed with MCOD activity, which is sensitive to induction by both PB and β-NF, in both studies. This may be due to the greater similarity

between the constitutive isoenzymes which have a role in the metabolism of 7-EC and of the *in-vivo* markers. It is conceivable that the use of substrates with even greater specificity for particular isoenzymes will improve upon the correlations observed here.

Studies with DEHP indicated it to be a weak inducer of antipyrine elimination, affecting the hydroxylation pathways in preference to the N-demethylation pathway. The induction process occurred slowly, requiring seven daily doses before maximal effects were observed, and its effects were only in evidence following administration of a relatively high dose of DEHP (2g/Kg). The weak effects that DEHP administration had on the *in-vitro* parameters, ECOD and EROD activities confirmed its weakness as an inducer of the isoenzymes involved in their metabolism. The potent induction of lauric acid hydroxylase activity in liver microsomes from rats pretreated with DEHP, confirmed DEHP to be effective as a specific inducer of P450IV. These data indicate that P450IV has minimal involvement in the metabolism of antipyrine. Another finding from the DEHP study was the observation that the corn oil diluent had weak inductive properties. Corn oil (3ml/Kg) produced a doubling in EROD and ECOD activities and an increase in LAH activity of of about 50%. The effects of corn oil and DEHP appeared to be additive. It was originally intended that DEHP would be used as a third type of inducing agent in the *in-vitro-in-vivo* correlation studies. The lack of a suitable *in-vivo* compound with sensitivity towards the levels of P450IV isoenzymes induced by DEHP, however, precluded such studies.

Inhibition of drug metabolism can be assessed by both *in-vitro* and *in-vivo* methods, but few studies have attempted to investigate the potency of an inhibitor by a combination of such methods. Although the inhibition of drug metabolism by ketoconazole has been established *in-vitro* by other investigators, its inhibitory potency *in-vivo* was unclear. It was the intention of these studies, therefore, to compare

the inhibitory potency of ketoconazole both *in-vitro* and *in-vivo* with that of cimetidine.

In-vitro studies established the value of K_i determinations over IC_{50} determinations in the comparison of inhibitory potency, the estimates of the latter being dependent, on the substrate concentration used in the assay. The use of microsomes from induced animals in the cimetidine studies, produced an even greater dependance of IC_{50} on substrate concentration. The inhibition of two enzyme activities was determined in control microsomes for both ketoconazole and cimetidine and in both instances, ketoconazole was about 100 times more potent an inhibitor than cimetidine.

The *in-vivo* studies were carried out in two parts. Firstly, the effects of single dose administration of ketoconazole on the CER parameters were determined following (N-methyl-^{14}C)-antipyrine administration. The effects of ketoconazole on the antipyrine breath test data were quite dramatic. The shape of the CER-time curve was altered in such a way that half-lives for antipyrine produced by various doses of ketoconazole could not be determined. However a marked dose-dependancy was observed in the decrease in CER_{max} and the increase in t_{max}. The appearance of a terminal phase at the lower ketoconazole doses, similar in half-life to that observed in controls, suggested that ketoconazole was being rapidly eliminated during the time course of the experiment. In order to obtain an estimate of ketoconazole inhibitory potency, a model was fitted to the data, using a nonlinear regression program, which took into consideration the fact that ketoconazole levels were not constant throughout the experiment.

Since the elimination of ketoconazole was extremely rapid even following ip administration ($t_{½}$ - 120min), it was necessary to carry out a steady state infusion study to investigate the relationship between ketoconazole plasma concentration and inhibition of antipyrine

elimination. The lack of pharmacokinetic data for ketoconazole in rats, in particular following iv administration, necessitated pharmacokinetic investigations. The pharmacokinetic parameters determined in these preliminary studies were used to determine suitable ketoconazole bolus doses and infusion rates for the steady state inhibition study. Although dose-dependent kinetics of ketoconazole were identified and resulted in the production of much higher concentrations of ketoconazole than predicted, it was not considered necessary to adjust the dosage regimen since steady state conditions were in operation. Allowance was made for the nonlinearity in the choice of regimen so that a wide range of ketoconazole steady state concentrations was achieved. The results of the antipyrine/ketoconazole interaction confirmed ketoconazole to be a potent inhibitor of antipyrine metabolism. Models fitted to the antipyrine clearance data indicated the K_i of ketoconazole to be about 1mg/L. Similar estimates were derived for each of the metabolite formation clearances which indicated that ketoconazole was non-selectively inhibiting all three major pathways. However although the inhibition reached a maximum at about 5mg/L, antipyrine clearance was only inhibited by about 55%. This indicated that about 50% of the isoenzymes involved in antipyrine metabolism were resistant to inhibition by ketoconazole.

It is clear from both the induction and inhibition studies carried out in this thesis that *in-vivo* and *in-vitro* approaches of determining drug metabolizing ability are complementary. *In-vitro* methods have the advantage that they allow a direct measurement of drug metabolizing ability uncomplicated by non-enzymic influences. However *in-vivo* determinations are required to confirm the relevance of these effects. Possession of both *in-vitro* and *in-vivo* data provides not only a more detailed description of the phenomenon under study, but allows a further comprehension of the intricacies. When dealing with such a complex

system as the cytochrome P-450 family of enzymes, such approaches are vital.

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APPENDIX

Table A.1 Individual Antipyrine Pharmacokinetic Rat Data For the Correlation Studies following β-NF Induction (section 4.4)

Pretreatment (mg/Kg)	Clearance ml/min/SRV	Half-life min	Volume of Distribution L/SRV	AUC mg x min/L
CO	1.64	78.5	0.19	7903
CO	1.52	80.7	0.18	8059
CO	1.79	107.0	0.28	4502
CO	2.09	67.3	0.20	5952
CO	2.30	79.3	0.26	5659
β-NF (3)*	3.74	51.0	0.28	3345
β-NF (3)	3.96	34.5	0.20	3198
β-NF (5)	4.11	40.8	0.24	2980
β-NF (5)	4.92	43.3	0.31	2574
β-NF (5)	3.51	49.2	0.25	3467
β-NF (7)	2.47	51.7	0.18	5075
β-NF (7)	4.65	30.0	0.21	2624
β-NF (7)	3.72	58.2	0.31	3364
β-NF (10)	4.09	31.4	0.19	3048
β-NF (20)	6.43	25.1	0.23	1914
β-NF (100)	6.51	21.3	0.20	1968

* dose

Table A.2 Individual Theophylline Pharmacokinetic Rat Data from the Correlation Studies following β-NF Induction (section 4.4)

Pretreatment (mg/Kg)	Clearance ml/min/SRV	Half-life min	Volume of Distribution L/SRV	AUC mg x min/L
CO	1.11	138.9	0.22	1705
CO	0.98	163.1	0.23	1901
CO	0.96	208.8	0.29	1233
CO	1.12	135.1	0.22	1680
CO	1.38	124.4	0.25	1383
β-NF (3)*	2.47	49.9	0.18	750
β-NF (3)	1.77	82.7	0.21	1064
β-NF (5)	3.83	40.3	0.22	475
β-NF (5)	3.60	37.7	0.20	523
β-NF (5)	3.28	53.7	0.25	562
β-NF (7)	2.63	58.2	0.22	714
β-NF (7)	4.40	30.4	0.19	432
β-NF (7)	2.12	81.7	0.25	893
β-NF (10)	4.60	29.1	0.19	409
β-NF (20)	7.25	18.2	0.19	254
β-NF (100)	10.63	19.0	0.29	179

* dose

Table A.3 Individual Antipyrine Formation Clearance Rat Data for the Correlation Studies following β-NF Induction (section 4.4)

Pretreatment	Formation Clearances		
(mg/Kg)	CL(4H) ml/min/SRW	CL(3H) ml/min/SRW	CL(H) ml/min/SRW
CO	0.11	0.10	0.09
CO	0.11	0.10	0.09
CO	0.27	0.20	0.17
CO	0.29	0.26	0.17
CO	0.36	0.27	0.23
β-NF (3)*	0.53	0.32	0.39
β-NF (3)	0.41	0.43	0.37
β-NF (5)	0.62	0.25	0.48
β-NF (5)	0.66	0.30	0.59
β-NF (5)	0.54	0.27	0.48
β-NF (7)	0.47	0.13	0.26
β-NF (7)	1.06	0.80	0.64
β-NF (7)	0.55	0.32	0.40
β-NF (20)	1.35	0.24	0.84
β-NF (100)	1.45	0.22	0.81

* dose

Table A.4 Individual *In-vitro* Rat Data for the Correlation Studies following β-NF Induction (section 4.4)

Pre-treatment (mg/Kg)	*MP	LW/BW	*P-450	*ECOD	*MCOD	*EROD	*ALE
CO	39.9	4.2	0.364	0.376	0.218	0.059	2.706
CO	46.6	4.3	0.372	0.322	0.185	0.051	2.407
CO	42.4	3.9	0.374	0.261	0.176	0.079	2.173
CO	43.9	4.8	0.411	0.344	0.203	0.052	2.349
CO	30.3	5.1	0.348	0.296	0.176	0.039	1.982
β-NF(3)*	39.0	4.9	0.458	1.258	0.288	0.931	2.574
β-NF(3)	40.4	4.9	0.460	0.800	0.323	0.417	3.031
β-NF(5)	38.8	4.5	0.595	1.245	0.387	2.299	2.207
β-NF(5)	42.9	5.0	0.636	0.881	0.364	1.382	2.895
β-NF(5)	49.2	4.3	0.755	0.877	0.328	1.514	2.506
β-NF(7)	33.9	4.8	0.527	1.983	0.207	1.074	1.725
β-NF(7)	44.2	4.5	0.488	2.170	0.305	1.445	2.450
β-NF(7)	39.2	4.8	0.527	0.858	0.263	0.377	2.179
β-NF(10)	67.7	4.4	0.746	2.856	0.347	4.701	2.267
β-NF(20)	41.4	5.2	0.691	3.372	0.301	3.188	1.706
β-NF(100	60.4	5.5	0.922	4.451	0.242	5.636	1.259

* dose
* MP = microsomal protein (mg/ml)
* nmol/mg microsomal protein
* nmol/min/mg microsomal protein

Table A.5 Individual Antipyrine Pharmacokinetic Rat Data For the Correlation Studies following PB Induction (section 4.5) (antipyrine/theophylline study)

Pretreatment (mg/Kg)	Clearance ml/min/SRW	Half-life min	Volume of Distribution L/SRW	AUC mg x min/L
Saline	2.10	91.8	0.28	6102
Saline	1.79	80.0	0.21	7019
Saline	1.86	90.4	0.24	6706
PB (5)*	3.05	52.5	0.23	4092
PB (5)	1.45	87.1	0.18	8631
PB (5)	4.28	38.7	0.24	2866
PB (10)	3.43	51.3	0.25	3628
PB (10)	3.15	52.5	0.24	3872
PB (20)	1.58	93.4	0.21	7637
PB (20)	3.74	52.3	0.28	3282
PB (20)	4.54	42.5	0.28	2717
PB (30)	4.10	38.1	0.23	3004
PB (80)	3.09	54.6	0.24	4010
PB (80)	3.37	50.4	0.25	4475
PB (100)	2.15	79.9	0.25	5671

* dose

Table A.6 Individual Theophylline Pharmacokinetic Rat Data For the Correlation Studies following PB Induction (section 4.5) (antipyrine/theophylline study)

Pretreatment mg/Kg	Clearance ml/min/SRW	Half-life min	Volume of Distribution L/SRW	AUC mg x min/L
Saline	0.82	176.4	0.21	2334
Saline	0.89	169.9	0.22	2114
Saline	0.76	173.5	0.19	2453
PB (5)*	1.08	141.7	0.22	1740
PB (5)	0.82	177.3	0.21	2282
PB (5)	1.80	70.4	0.18	1025
PB (10)	0.96	147.5	0.20	1947
PB (10)	1.16	130.5	0.22	1570
PB (20)	0.52	361.0	0.27	3503
PB (20)	1.17	131.6	0.22	1568
PB (20)	1.17	112.4	0.18	1575
PB (30)	1.44	126.0	0.26	1283
PB (80)	0.82	175.1	0.21	2261
PB (80)	1.22	143.0	0.25	1855
PB (100)	0.74	181.5	0.19	2466

* dose

Table A.7 Individual Antipyrine Metabolite Clearance Rat Data for the Correlation Studies (section 4.5) following PB Induction (Antipyrine/Theophylline Study)

Pretreatment	Formation Clearances		
(mg/Kg)	CL(4H) ml/min/SRW	CL(3H) ml/min/SRW	CL(N) ml/min/SRW
Saline	0.16	0.16	0.13
Saline	0.26	0.25	0.17
Saline	0.27	0.26	0.20
PB (5)*	0.42	0.43	0.28
PB (5)	0.14	0.12	0.08
PB (5)	0.55	0.54	0.40
PB (10)	0.26	0.22	0.24
PB (10)	0.26	0.21	0.19
PB (20)	0.22	0.12	0.10
PB (20)	0.44	0.37	0.33
PB (20)	0.52	0.48	0.42
PB (30)	0.69	0.46	0.36
PB (60)	0.35	0.21	0.21
PB (60)	0.46	0.28	0.24
PB (100)	0.19	0.13	0.11

* dose

Table A.8 Individual *In-vitro* Rat Data from the Correlation Studies following PB Induction (section 4.5) (antipyrine/theophylline study)

Pre-treatment (mg/Kg)	[a]MP	LW/BW	[b]P-450	[c]ECOD	[c]MCOD	[c]EROD	[c]ALE
Saline	49.6	4.5	0.344	0.218	0.134	0.028	2.281
Saline	42.9	4.2	0.366	0.390	0.205	0.053	1.598
Saline	34.1	4.6	0.370	0.271	0.196	0.045	1.959
PB (5)[a]	41.3	4.6	0.583	0.720	0.378	0.064	2.317
PB (5)	45.0	4.1	0.541	0.525	0.263	0.054	1.796
PB (5)	35.2	5.5	0.566	0.652	0.404	0.058	2.730
PB (10)	46.6	4.2	0.569	0.909	0.419	0.073	3.893
PB (10)	38.8	4.0	0.571	0.554	0.295	0.076	1.758
PB (20)	44.3	3.7	0.588	0.941	0.452	0.105	2.236
PB (20)	38.6	5.2	0.727	0.678	0.444	0.057	2.768
PB (20)	42.0	4.4	0.665	0.891	0.508	0.039	3.072
PB (30)	46.8	4.8	0.695	1.136	0.596	0.108	3.002
PB (80)	47.4	4.3	0.615	1.056	0.705	0.059	2.542
PB (80)	49.3	4.3	0.721	1.088	0.718	0.071	3.053
PB (100)	47.4	4.2	0.531	0.575	0.254	0.047	1.876

[a] dose
[b] MP = microsomal protein (mg/ml)
[b] nmol/mg microsomal protein
[c] nmol/min/mg microsomal protein

Table A.9 Individual Antipyrine Pharmacokinetic Rat Data for the Correlation Studies following PB Induction (section 4.5) (Antipyrine/Tolbutamide study)

Pretreatment mg/Kg	Clearance ml/min/SRW	Half-life min	Volume of Distribution L/SRW	AUC mg x min/L
Saline	2.30	70.8	0.24	5306
Saline	2.06	75.6	0.22	6143
Saline	2.30	73.3	0.24	5390
PB (2.5)*	2.63	47.2	0.18	4670
PB (2.5)	3.55	49.3	0.25	3333
PB (5)	2.78	57.8	0.23	4273
PB (7.5)	3.22	53.0	0.25	3704
PB (7.5)	2.78	44.2	0.18	4242
PB (10)	4.40	33.8	0.21	2594
PB (10)	2.54	67.0	0.25	4603
PB (15)	3.69	51.1	0.27	3375
PB (15)	4.30	36.1	0.22	2829
PB (20)	2.88	47.9	0.20	4386
PB (20)	4.72	38.3	0.26	2649
PB (80)	3.89	34.1	0.19	3102
PB (80)	6.27	27.4	0.21	2205

* dose

Table A.10 Individual Tolbutamide Pharmacokinetic Rat Data for the Correlation Studies following PB Induction (section 4.5) (Antipyrine/Tolbutamide study)

Pretreatment mg/Kg	Clearance ml/min/SRW x 10^2	Half-life min	Volume of Distribution L/SRW x 10^2	AUC mg x min/L
Saline	9.85	202.6	2.88	24811
Saline	15.35	167.5	3.71	16481
Saline	14.35	172.4	3.57	17307
PB (2.5)*	17.70	114.2	2.92	13857
PB (2.5)	16.62	156.5	3.75	14233
PB (5)	15.25	158.7	3.49	15574
PB (7.5)	18.32	128.2	3.39	13033
PB (7.5)	17.95	136.9	3.55	13158
PB (10)	26.81	93.5	3.62	8508
PB (10)	15.85	177.0	4.05	14744
PB (15)	18.84	126.3	3.43	13201
PB (15)	26.04	93.9	3.53	9342
PB (20)	20.98	123.3	3.73	12056
PB (20)	25.02	104.3	3.77	9998
PB (80)	27.73	88.5	3.54	8697
PB (80)	37.85	63.8	3.48	6140

* dose

Table A.11 Individual *In-vitro* Rat Data from the Correlation Studies (section 4.5) following PB Induction (antipyrine/tolbutamide study)

Pre-treatment (mg/Kg)	[a]MP	LV/BV	[b]P-450	[c]ECOD	[d]MCOD	[e]HROD	[d]ALH
Saline	38.1	4.7	0.418	0.431	0.353	-----	1.886
Saline	40.3	4.3	0.527	0.412	0.212	-----	1.408
Saline	47.4	4.5	0.643	0.414	0.206	-----	1.556
PB (2.5)[a]	40.7	4.2	0.544	0.524	0.316	-----	2.079
PB (2.5)	43.7	4.3	0.538	0.512	0.310	-----	2.379
PB (5)	34.6	4.3	0.571	0.658	0.456	-----	2.865
PB (7.5)	33.5	4.3	0.896	0.897	0.610	-----	2.307
PB (7.5)	44.3	4.0	0.588	0.634	0.374	-----	1.722
PB (10)	40.6	3.9	0.758	0.910	0.662	-----	2.109
PB (10)	42.8	4.3	0.709	0.518	0.306	-----	1.031
PB (15)	34.8	4.6	0.648	0.827	0.555	-----	1.616
PB (15)	41.5	4.3	0.989	1.388	0.683	-----	3.064
PB (20)	39.2	5.0	0.901	0.893	0.559	-----	1.953
PB (20)	46.8	4.5	0.852	1.033	0.647	-----	2.553
PB (80)	35.9	5.5	0.973	1.328	0.929	-----	2.521
PB (80)	48.8	5.5	1.060	1.904	1.029	-----	3.426

[a] dose
[b] MP = microsomal protein (mg/ml)
[c] nmol/mg microsomal protein
[d] nmol/min/mg microsomal protein

Table A.12 Pharmacokinetic parameters of antipyrine in the presence of various steady state concentrations of ketoconazole for each individual rat (section 5.5)

KETOCONAZOLE STEADY STATE CONCENTRATION (mg/L)	CLEARANCE (ml/min/SRW)	HALF-LIFE (min)	VOLUME OF DISTRIBUTION (L/SRW)
0	1.54	136.6	0.31
0	1.99	108.8	0.31
0	2.13	101.3	0.31
0	2.15	83.2	0.26
0	2.36	90.9	0.31
0	2.36	83.8	0.29
0	2.54	93.6	0.31
1.24 (0.12)	1.80	114.3	0.30
1.34 (0.08)	1.73	134.0	0.34
1.44 (0.62)	1.36	132.0	0.26
1.46 (0.26)	1.10	173.2	0.28
1.83 (1.24)	1.03	206.1	0.31
1.91 (0.69)	1.30	306.3	0.57
2.92 (0.68)	1.23	194.9	0.35
3.09 (0.50)	1.62	133.4	0.31
3.64 (0.04)	0.96	196.4	0.27
4.33 (1.14)	1.30	179.6	0.34
4.49 (0.79)	1.08	190.5	0.30
4.59 (0.55)	1.35	157.2	0.31
6.40 (1.57)	1.27	186.1	0.34
6.68 (0.91)	0.77	250.9	0.28
6.83 (1.05)	1.00	198.8	0.28
7.23 (1.04)	0.84	247.4	0.30
7.28 (1.46)	0.74	264.0	0.28
7.76 (1.05)	1.02	173.6	0.26
7.91 (2.03)	1.10	204.6	0.32
9.76 (0.49)	0.89	240.7	0.31
11.47 (3.11)	1.12	157.2	0.26
12.15 (1.84)	0.83	255.7	0.31
12.28 (1.69)	0.78	279.6	0.32
14.88 (3.15)	1.20	189.0	0.33
15.06 (7.40)	0.92	253.8	0.34
16.73 (8.28)	0.69	274.4	0.27

Table A.13 Individual Rat antipyrine metabolite formation clearance data for section 5.5.

KETOCONAZOLE STEADY STATE CONCENTRATION (mg/L)	FORMATION CLEARANCE (ml/min/SRW)			RENAL CLEARANCE (ml/min/SRW)
	4OH-	NOR-	3OHMe-	
0	0.35	0.20	0.21	0.13
0	0.53	0.33	0.37	0.07
0	0.39	0.22	0.39	0.08
0	0.46	0.25	0.43	0.04
0	0.37	0.32	0.56	0.08
0	0.58	0.31	0.45	0.06
0	0.36	0.29	0.30	0.07
1.24 (0.12)	0.43	0.20	0.45	0.05
1.34 (0.08)	0.37	0.16	0.38	0.05
1.44 (0.62)	0.28	0.14	0.19	0.07
1.46 (0.26)	0.18	0.17	0.19	0.02
1.83 (1.24)	0.13	0.13	0.15	0.05
1.91 (0.69)	0.16	0.12	0.20	0.02
2.92 (0.68)	0.18	0.10	0.16	0.02
3.09 (0.50)	0.38	0.19	0.37	0.10
3.64 (0.04)	0.14	0.09	0.17	0.06
4.33 (1.14)	0.37	0.15	0.34	0.09
4.49 (0.79)	0.16	0.10	0.14	0.04
4.59 (0.55)	0.42	0.18	0.28	0.06
6.4 (1.57)	0.29	0.13	0.23	0.09
6.68 (0.91)	0.13	0.06	0.16	0.05
6.83 (1.05)	0.23	0.11	0.20	0.06
7.23 (1.04)	0.23	0.08	0.16	0.07
7.28 (1.46)	0.14	0.07	0.10	0.05
7.76 (1.05)	0.20	0.11	0.20	0.06
7.91 (2.03)	0.19	0.07	0.15	0.04
9.76 (0.49)	0.28	0.11	0.19	0.08
11.47 (3.11)	0.30	0.14	0.29	0.06
12.15 (1.84)	0.21	0.13	0.21	0.06
12.28 (1.69)	0.17	0.09	0.14	0.05
14.88 (3.15)	0.24	0.14	0.27	0.08
15.06 (7.40)	0.16	0.08	0.13	0.05
16.73 (8.28)	0.10	0.05	0.12	0.06