THE ASSESSMENT OF FLAVIN-CONTAINING MONOOXYGENASE ACTIVITY IN INTACT ANIMALS*

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SUMMARY

A large number of drug metabolising enzymes with different substrate specificities and induction and inhibition characteristics have been described, suggesting that specific test drugs, i.e. probes, should be used for assessing the activity of distinct metabolising enzymes. The flavin-containing monooxygenase (FMO) and cytochrome P-450 (P-450) are the two main microsomal enzyme systems involved in the oxidation of xenobiotics. FMO is present in liver and other tissues of most vertebrates. It catalyses the oxidation of a wide range of xenobiotics, especially soft nucleophiles bearing nitrogen and sulphur centres. There is substantial information on both *in vitro* and *in vivo* probes for cytochrome P-450. For example antipyrine has been widely used for assessing the activity of P-450 *in vivo* by utilising pharmacokinetic parameters as indices of enzyme activity. In more recent years, isozyme specific probes have also been developed for some of the P-450s. Whereas a number of substrates are available for measuring FMO activity *in vitro* (e.g. *N,N*-dimethylaniline), probes for assessing FMO activity *in vivo* are limited. In this review a background to the use of *in vitro* and *in vivo* probes for assessing the activity of FMO is presented, and approaches and criteria for development of potential pharmacokinetic probes for FMO are described. Preliminary data on the development of ethyl methyl sulphide (EMS) and trimethylamine (TMA) as potential pharmacokinetic probes for assessing FMO activity in rats are discussed in detail. Clinical implications of modulation of FMO activity are discussed, and arguments presented as to why the development of FMO probes for use in man will be useful additions to the range of other compounds available for assessment of liver metabolic function.

1. INTRODUCTION

1.1. Background

The flavin-containing monooxygenase (FMO) and cytochrome P-450 are the two main microsomal enzyme systems involved in the oxidation of xenobiotics. A number of investigators had observed that the enzyme system catalysing the oxidation of nucleophilic tertiary and secondary amines exhibited properties that were distinct from the cytochrome P-450 system /1/. These early observations were later

confirmed in pioneering studies by Ziegler and his team, who purified and characterised a microsomal flavin-containing monooxygenase from porcine liver that catalyses the oxidation of nucleophilic organic nitrogen, sulphur, selenium and phosphorus compounds (see /2/ for review). Enzyme systems similar in composition and mechanism to the now widely studied porcine liver microsomal flavin enzyme are usually referred to as the flavin-containing monooxygenases (FMOs). Unlike cytochrome P-450, the flavin-containing monooxygenase possesses only a flavin adenine dinucleotide as a prosthetic group. In mammals, the flavin-containing monooxygenase has apparently evolved to detoxify soft nucleophiles and other non-nutrients that are so abundant in plant sources /3/. The unique catalytic mechanism of FMO is ideally suited for this role /4-7/.

Whereas FMO substrates differ widely in structural configurations, they have one feature in common in that they are all nucleophiles, and only compounds bearing a polarisable electron rich centre are potential substrates. It is now clear that compounds that are readily oxidised by organic hydroperoxides are also readily oxidised by FMO, provided they can gain access to the enzyme bound flavin-hydroperoxide at the enzyme active site. These flavoproteins appear to discriminate readily between essential and exogenous nucleophiles, since nucleophiles that resemble physiologically useful metabolites are excluded from the active site of FMO by steric and ionic requirements. A comprehensive list of both synthetic and naturally occurring xenobiotics that are FMO substrates has been covered by current reviews /4-7/. Recent reports demonstrate that some species contain more than one isoform of the flavin-containing enzyme /8-12/. An FMO gene family composed of at least five members (i.e. five different FMO isoforms) has been described /12/, and a nomenclature for the FMO gene family based on amino acid sequence identities has been proposed. The five members described to date are named FMO1, FMO2, FMO3, FMO4 and FMO5 /12/. As in the case of P-450s, it would seem that there are several FMOs with overlapping substrate specificities, and that the relative proportions of these isoforms may vary in different tissues within and between species /82,83,90,91/.

1.2. Induction and inhibition of P-450 and FMO

Induction and inhibition of drug metabolising enzymes provides an important tool in elucidating the role of these enzyme systems in biotransformation reactions. Induction was first discovered during the

early studies of the metabolism of carcinogens /13/ and on the tolerance of barbiturates /14/. Following these initial findings, many drug metabolising enzymes, particularly isozymes of cytochrome P-450, have been found to be inducible by xenobiotics /15/. Many inhibitors of oxidative drug metabolism have also been described /16/. The nature of the metabolising enzyme reaction cycle (e.g. cytochrome P-450 cycle) clearly shows a number of potential points of interaction with inhibitors of drug oxidations.

It has generally been assumed that FMO is not inducible since attempts to demonstrate induction in animals pretreated with exogenous substances have been unsuccessful /17/. Also, there are no selective mechanism based FMO inhibitors at present, probably due to the fact that the substrates for FMO are metabolised by an ionic as opposed to a radical mechanism /18/. However, FMO has been shown to be under hormonal, developmental and possibly nutritional control. Recently, a significant loss of FMO activity has been observed in rats fed orally or parenterally with chemically defined diets essentially free from xenobiotics /19,20/, implying that FMO may normally be in a state of full induction.

Experimental protocols for assessing the role and activity of the flavin-containing monooxygenase in the metabolism of soft nucleophiles *in vivo* are limited. This is probably due to lack of specific inhibitors or inducers for modulating the activity of FMO *in vivo*, since modulation of FMO activity would be needed to validate potential *in vivo* FMO probes. Although methimazole has been used as an inhibitor of FMO, the data from such studies have to be interpreted with caution since methimazole is metabolised to a reactive intermediate that leads to the loss of glutathione and oxidation of protein thiols /21/, and may therefore be affecting more than one enzyme system. Dimethyl sulphoxide (DMSO) has been suggested as a potential candidate for manipulating the activity of FMO *in vivo* /6/. DMSO is a substrate for FMO, although its K_m is around 3-4 mM (compared to low mM or μM for many better substrates). This compound, in fairly high doses, has been demonstrated to decrease dramatically the ratio of trimethylamine *N*-oxide (TMAO) to trimethylamine (TMA) in urine from rats dosed with TMA /6/. DMSO, like methimazole, may not be selective in its action, and indeed it has been shown to induce certain P-450s /41/.

FMO has been reported to be regulated developmentally in a number of species; examples include sex differences and variations with age, oestrus cycle or pregnancy /22/. Changes in activity of liver

FMO of mice and guinea-pigs as a result of food restriction have also been reported /23/. An earlier study /24/ had also reported the non-specific loss of liver microsomal enzymes in rats fed intravenously, but critical evaluation of this work has not been possible due to lack of experimental details reported. Recently, the selective loss of FMO activity in rats on chemically defined diets has been reported /19,20/, and it would therefore appear that FMO is under developmental, hormonal *and* nutritional control, and indeed genetic control (see below).

1.3. Assessing FMO activity *in vivo*

The contribution of FMO to the metabolism of xenobiotics in the intact animal is often difficult to assess unambiguously by measuring metabolic end products. The primary reason for this is that oxidation products generated invariably undergo metabolic reduction or further metabolism, and only the proportion that escapes such metabolism is excreted /4,25/. For example, attempts to use urinary metabolite levels of typical *in vitro* substrates of FMO (e.g. *N,N*-dimethylaniline) to assess enzyme activity in intact animals have often been associated with difficulties due to the oxidation-reduction cycling that occurs *in vivo*. Consequently, velocity of reactions catalysed by the FMO relative to other competing pathways *in vivo* cannot be determined categorically by measuring metabolic end products in bile or urine /4/. Other approaches to assess the activity of FMO *in vivo* are therefore required. Correlation between *in vitro* and *in vivo* markers of FMO activity is yet another area that has not received significant attention to date from research workers. Therefore, there is a need to develop systematically *in vivo* pharmacokinetic probes for monitoring the activity of FMO. This can be readily achieved in experimental animals by studying the pharmacokinetics of specific FMO substrates and by manipulating the cellular environment of FMO non-invasively, by feeding chemically defined diets to animals, to validate the probes under development. Pharmacokinetic probes for monitoring the activity of this enzyme in intact animals have been recently described /20,26, 27/ utilising this approach.

The ability to correlate *in vivo* pharmacokinetic and metabolic data with *in vitro* metabolic data has been emphasized in recent years. Both quantitative and qualitative data obtained from *in vitro* studies should be relevant to the situation *in vivo* /28/. The rate of formation of a given metabolite *in vivo*, for example, can be predicted from *in vitro*

studies. When a drug is eliminated entirely by metabolism, clearance from blood is equivalent to metabolic clearance and for a drug that forms more than one metabolite, hepatic intrinsic clearance (Cl_{int}) will be the sum of the clearances of individual metabolites which can be expressed in terms of their Michaelis-Menten constants (K_m and V_{max}) according to the equation:

$$Cl_{int} = \sum_{i=1}^{n} \frac{V_{m,i}}{K_{m,i}}$$

Therefore, an increase in drug metabolising enzyme activity will increase the clearance of the drug and *vice versa*. Thus, any change in enzyme activity *in vivo* will be reflected by a change in the blood levels of the parent drug, and indeed drug clearance has been used as a marker of enzyme activity in intact animals /29/. Whereas these concepts have been applied to the development of *in vivo* probes for P-450 isozymes, it is only recently that these have been considered for measurement of FMO activity in intact animals /20,26,27/.

Metabohc FMO mediated *N*- and *S*-oxygenations in particular represent important routes of metabolism for a large number of pharmacologically potent drugs and other foreign compounds /4,5,7, 30-32/. Therefore there is an urgent need to develop reliable *in vivo* probes for measuring the capacity of FMOs in humans. It is obvious that any changes in the activity of FMOs, through environmental, physiological, nutritional or genetic factors, would have an effect on drug disposition, and an effect ultimately on drug pharmacology and/or toxicology. This review presents background on the use of *in vitro* and *in vivo* probes for assessing the activities of P-450 and FMO, and describes approaches and criteria for potential pharmacokinetic probes for FMO. Preliminary data from the present authors' laboratory on the development of ethyl methyl sulphide (EMS) and trimethylamine (TMA) as potential probes for assessing FMO activity in intact animals are also described.

2. *IN VITRO* AND *IN VIVO* PROBES FOR CYTOCHROME P-450

2.1. *In vitro* probes for cytochrome P-450

The assessment of drug metabolising capacity using *in vitro* techniques is probably the most direct and easy method, provided a good correlation with the *in vivo* situation can be established. A large number of drugs and other xenobiotics undergo oxidative reactions in mammalian tissues that are mediated by the cytochrome P-450-dependent system, and some of these compounds have been adapted as *in vitro* probes for the P-450 system. For example, convenient and reproducible assessment of P-450 activity *in vitro* can be achieved by studying 4-hydroxylation of aniline /33/, *N*-demethylation of aminopyrine /34/, *O*-demethylation of 4-nitroanisole /35/, *O*-deethylation of 7-ethoxycoumarin /36/, ring hydroxylation of benzo[*a*]pyrene /37/ and *O*-dealkylation of a series of alkyl-substituted resorufins /38/. The resorufins have found increasing favour in recent years, not only because of the applicability of a sensitive fluorometric assay, but also because the different alkyl-substituted resorufins are substrates for different cytochrome P-450 isoforms, e.g. ethoxyresorufin is metabolised by CYP1A1, and pentoxyresorufin is metabolised by CYP2B1 /39/. A number of other *in vitro* isozyme specific P-450 probes have been developed in recent years /40-43/.

2.2. *In vivo* probes for cytochrome P-450

Approaches used to study *in vivo* drug metabolism include measurement of drug and metabolites in urine and in blood over an extended period of time. Pharmacokinetics is particularly important for the study of drug metabolism in man. Many *in vivo* probes have been described for assessing the activity of cytochrome P-450 in intact animals, especially man (Table 1). The measurement of clearance of the probe from blood or other relevant body fluid is the ideal approach employed as a marker for drug metabolising activity *in vivo*. A number of criteria are used before clearance can be used as an indicator of drug metabolising activity /44/. These include:

- the probe should be rapidly and ideally completely and reproducibly absorbed. This is particularly important for human studies, when oral dosing is the norm
- the probe should not bind to plasma proteins extensively

TABLE 1

In vivo probes used for assessing cytochrome P-450 activity in man by measuring blood/plasma clearance

Substrate	Reference
Antipyrine	46
Phenacetin	50
Caffeine	54
Theophylline	57
Paracetamol	56
Debrisoquine	51
Sparteine	51
Diazepam	55
Mephenytoin	52
Codeine	59
Isoniazid	60
Warfarin	58

- the liver should be the predominant organ for metabolising the probe
- renal clearance of the probe and its metabolites should be measurable
- the probe should not be eliminated too quickly, to enable the clearance to be accurately measured
- the assay method for the probe and metabolites should be sensitive and specific, and ideally the assay should be capable of being automated, for ease of use in clinical laboratories
- the probe should be safe.

The first 'probe' that satisfied most of these criteria was antipyrine /45-49/. However antipyrine clearance does not always correlate well with metabolism of other drugs /44/. This is due to the involvement of more than one isoform of P-450 in antipyrine metabolism and the production of at least three metabolites. The rate of metabolism of antipyrine as measured by its intrinsic clearance is thus an overall index of oxidising activity of the different P-450 isoforms involved in its metabolism, rather than the activity of a specific P-450 isoform. Direct

correlations of drug clearances are therefore only possible when the two drugs are metabolised by the same isozyme(s).

Other model substrates that have been utilised to assess cytochrome P-450 activity *in vivo* are listed in Table 1. This list is by no means complete, since the number of probes in use, or proposed, is quite large. Phenacetin, caffeine and theophylline are believed to be substrates for CYP1A2 and thus act as specific markers of the activity of this isozyme /50/. Sparteine and debrisoquine have also been used as isozyme specific probes for CYP2D6, using drug:metabolite ratio in urine as an indicator of enzyme activity /51/. Mephenytoin biotransformation has also been used as a marker of CYP2C9 /52/. Erythromycin has also been utilised in recent years with considerable success in the 'erythromycin breath test' to selectively measure CYP3A enzymes (see /53,110/ for authoritative reviews). Thus careful use of these probes, sometimes as 'cocktails', can yield useful information on the activity of cytochrome P-450 isozymes in intact organisms, particularly in man /53/. This discussion of P-450s is not comprehensive, but is presented merely to illustrate the point that at present there are a large number of probes/approaches for the measurement of P-450 activity *in vitro* and *in vivo*, but considerably fewer for the FMO enzymes.

3. *IN VITRO* AND *IN VIVO* PROBES FOR FMO

3.1. *In vitro* probes for FMO

The list of xenobiotics that are sufficiently nucleophilic to be readily metabolised by FMO is extensive; Table 2 lists some common substrates.

The measurement of FMO activity in microsomes or hepatocytes requires a) selection of a model compound that is selectively metabolised to a stable product by this enzyme system, and b) the development of a highly sensitive and specific assay system to quantify the very often low levels of metabolite formed. The *N*-oxygenation of *N,N*-dimethylaniline (DMA) has most commonly been used to estimate FMO activity in different tissues, species and cell types /6,64-66, and references cited therein/. The product (DMA *N*-oxide) has been measured colorimetrically /64/, or by gas chromatography (GC) or HPLC /65,79/. On the basis of studies with rat microsomes /98/ and with two rat hepatic purified cytochromes (CYP2B1, major phenobarbitone inducible form, and CYPIAI, major β-naphthoflavone inducible

form) /99/, it was clear that *N*-oxidation of *N,N*-dimethylaniline (at least in the rat) was mediated exclusively *via* the FMO, whereas *N*-demethylation was by a phenobarbitone-inducible P-450. *N,N*-Dimethylaniline is therefore potentially an excellent single model compound for monitoring the activities of both these monooxygenases; an estimate of *N*-oxidase activity reflecting the status of FMO, whereas *N*-demethylase activity would be a measure of the P-450 system /7/. This compound has successfully been used to measure FMO and P-450 activities in rat hepatocytes /65/, and the validity of the data confirmed using additional substrates (7-ethoxycoumarin for P-450, and tetrahydrothiophen for FMO).

TABLE 2

Some *in vitro* substrates for FMO

Substrate	Reaction type	Reference
Promethazine	Sulphoxidation	61
Chlorpromazine	Sulphoxidation	62
Imipramine	*N*-Oxidation	63
N,N-Dimethylaniline	*N*-Oxidation	64-66
Trimethylamine	*N*-Oxidation	66,86
Thiobenzamide	Sulphoxidation	67,68
Diethylsulphide	Sulphoxidation	69,70
Senecionine	Sulphoxidation	71
Ethyl methyl sulphide	Sulphoxidation	72
Tamoxifen	*N*-Oxidation	73
Nicotine	*N*-Oxidation	74
Pargyline	*N*-Oxidation	75
N-Ethyl-*N*-methylaniline	*N*-Oxidation	76
Cimetidine	Sulphoxidation	77,78
Guanethidine	*N*-Oxidation	93

In addition to *N,N*-dimethylaniline, a large number of other FMO substrates have been proposed as potential *in vitro* probes for estimating FMO activity. These include model thioether substrates /69,70,72,82,83/, *N*-alkyl-*N*-methylanilines /76,84,85/, the simple tertiary amine trimethylamine /66,86/, the tobacco alkaloid (*S*)-nicotine /74,78/, the drugs cimetidine /78/, (*D*)- and (*L*)-brompheniramine /87/,

(*E*)- and (*Z*)-zimeldine /87/ and guanethidine /93/, amongst many others (see Table 2). An interesting recent simple, rapid and sensitive assay has been described, which utilises benzydamine (BZY) *N*-oxidation, with HPLC fluorometric determination of the BZY *N*-oxide /88,89/. Since recent reports suggest that certain *S*- and *N*-oxygenations, previously thought to be pure FMO reactions, are also mediated partially by P-450s (e.g. /66,97/), considerable care should be exercised in choice of substrate and in experimental design (e.g. use of P-450 inhibitors to suppress P-450 mediated reactions).

Other alternative approaches that have commonly been used to assess FMO activity include following oxygen uptake polarographically in the presence of specific substrates, e.g. methimazole /80/, or following NADPH oxidation spectrophotometrically /81/. Although the last two methods are rapid and convenient, they lack specificity, and are only suitable for use with pure FMOs. The concentration of FMO in crude preparations can be estimated immunochemically, but requires antibodies to the various FMO isoforms /4/.

In view of the fact that the multiplicity of FMO is now well established /82,83,90,91/, the distinct substrate specificities of the known FMO isoforms needs comment in the context of developing specific probes. Rabbit lung flavin-containing monooxygenase (FMO2) readily catalyses the oxidation of primary alkylamines, whereas rat and porcine hepatic FMO (FMO1) oxidises only tertiary amine substrates. However, both enzymes share common substrates in *N,N*-dimethylaniline and trifluoperazine /4,92/. Isozyme-specific substrates would obviously be useful probes for assessing the activity of FMO *in vitro*, compared to the non-specific *N,N*-dimethylaniline. Unfortunately isozyme specific substrates that are selective for FMO isoforms are few at present. Detailed substrate specificity studies, particularly with homologous series of prochiral tertiary amines /84,85/ and prochiral sulphides /82,83/, using pure mammalian tissue FMOs, or *c*DNA-expressed FMO isoforms, may lead to the identification of isozyme-selective probes for microsomal FMOs. Our current studies (Hadley, Hutt and Damani, unpublished data) on the stereoselective or stereospecific *N*-oxidation of *N*-alkyl-*N*-methylanilines may be useful mechanistically, as it may be possible to differentiate between isoforms, and thus provide simple probes for their classification and for an understanding of their active site topology. Indeed several reports /94-96/ have suggested that subtle differences in structural elements control access of nucleophilic substrates into the binding channel of

each isoform. Therefore a judicious choice of sulphur- or nitrogen-containing congeners may facilitate a clearer understanding of the active site(s) of the tissue- and species-specific isoforms of the FMOs.

3.2. *In vivo* probes for FMO

Some of the problems associated with assessing FMO capacity *in vivo* using urinary drug:drug metabolite ratios have been addressed in Section 1.3. The view of the present authors /20,25-27/ is that development of *in vivo* pharmacokinetic probes based on blood/plasma measurements may overcome some of the drawbacks of such approaches, or pharmacokinetic methods may act as complementary methods, at least for systematic investigations of FMO regulation in animal species. Nonetheless, it is noted that the measurement of urinary excretion of a few drugs and their metabolites has been shown to be a valid indicator of FMO activity in certain circumstances, e.g. use of trimethylamine. Trimethylamine is metabolised by FMO to trimethylamine *N*-oxide (TMAO), and this metabolite can readily be measured in urine by GC. The ratio of trimethylamine/trimethylamine *N*-oxide (TMA/TMAO) in human urine has been used successfully to probe into the genetic condition trimethylaminuria or fish odour syndrome /100,101/. *N*-Oxidation of TMA is genetically determined, and exhibits polymorphism in a white Caucasian population. The ability to *N*-oxidise TMA derived from normal dietary sources was found to be skewed in terms of its population distribution when investigated in 169 British white individuals /100-103/. Subsequently, subjects identified as 'trimethylaminurics' and their family members were studied in more detail as carriers of this genetic defect using a trimethylamine load test /102/. It has been proposed that TMA *N*-oxidation is controlled by a single autosomal diallelic locus with one allele for rapid and extensive oxidation, and an uncommon variant allele for impaired metabolism (see /103/ for review). Urinary levels of TMA and TMAO have therefore successfully been used for assessing FMO capacity in humans. Urinary TMA/TMAO ratios have also been used in studies with rats, in which attempts were made to modulate FMO activity using high doses of DMSO (see Section 1.2).

N-Oxidation of nicotine isomers has been demonstrated to be mediated by purified porcine hepatic FMO (FMO1) /74/, and urinary levels of nicotine-1′-*N*-oxide have been used as a measure of FMO activity in chewers of nicotine gum /104/, and in subjects identified as sufferers of trimethylaminurea /105/. In the former study /104/, co-

administration of methimazole was shown to reduce the production of nicotine-1′-*N*-oxide, when compared with a control untreated group. In the latter study /105/ deficient nicotine *N*-oxidation was demonstrated in two sisters with trimethylaminurea. Thus there is scope for the use of this compound as an *in vivo* probe for FMO.

Apart from the use of TMA and nicotine as human *in vivo* FMO probes, albeit with measurement of metabolic end products in urine, human *in vivo* pharmacokinetic probes based on blood/plasma measurements are not yet available. Recently the effects of methimazole on the disposition kinetics of netobimin (NTB), a probenzimidazole compound which exerts its anthelmintic activity by cyclization into albendazole metabolites, have been described in cattle /106/. It was proposed that FMO is responsible for albendazole (ABZ) oxidation into albendazole sulphoxide (ABZSO), while cytochrome P-450 was responsible for the second and slower oxidation step to albendazole sulphone ($ABZSO_2$). The results of this study demonstrated that the co-administration of methimazole with parenterally or orally administered NTB in cattle results in significant changes in the pharmacokinetic behaviour of the ABZSO metabolite. The alteration in pharmacokinetic parameters for ABZSO could therefore be interpreted to reflect modulation of FMO activity by methimazole, at least for the first oxidation step of albendazole. Detailed pharmacokinetic studies with albendazole may be fruitful in developing an *in vivo* probe for FMO.

Apart from the above, very few other compounds have been described as *in vivo* FMO probes. Studies from the present authors' laboratory on TMA and ethyl methyl sulphide (EMS) as potential pharmacokinetic probes for FMO are discussed in more detail in Sections 4 and 5.

4. CRITERIA FOR DEVELOPING PHARMACOKINETIC PROBES FOR FMO

4.1. Background

The use of TMA and (*S*)-nicotine (with measurement of urinary levels of their *N*-oxides) and the potential use of albendazole (with blood/plasma level measurements) as *in vivo* probes for FMO has already been discussed in Section 3.2. This section deals with potential new *in vivo* pharmacokinetic probes based on blood/plasma

measurements. Two important considerations in developing a probe are: 1) careful choice of the substrate, and 2) choice of method for modulating FMO activity to test the validity of the probe. The criteria for *in vivo* probes as discussed in Section 2.2 are of course equally applicable to FMO probes. As indicated before, the activity of biotransformation enzymes in intact animals is preferably expressed by the clearance from blood of a specific substrate of a particular enzyme, or of one of its metabolites which may be an enzyme specific product.

4.2. Choice of *in vivo* markers for FMO

The choice of an *in vivo* marker for FMO should ideally be based on the knowledge of the relative participation of the various Phase I and Phase II enzymes (and their isoforms) in its biotransformation. The *in vivo* pharmacokinetic approach would require the administration of an FMO probe that is metabolised to an FMO-specific product (metabolite), and the monitoring of the levels of the parent compound and the metabolite in biofluid. Ideally the participation of other drug metabolising enzymes in the pathway of interest should be minimal. Therefore, the ideal probe should be converted primarily to a single enzyme specific product which does not readily undergo further biotransformation. Most current FMO substrates fall short of this ideal. However McManus *et al.* /93/ have postulated the use of the antihypertensive agent guanethidine as a potential probe for assessing the activity of FMO. They point out that guanethidine is converted primarily to its *N*-oxide by FMO, while the guaninidine moiety does not undergo metabolic transformation. Furthermore, guanethidine *N*-oxide is one of the few tertiary amine *N*-oxides that is not readily reduced or subject to further metabolism. However, there are disadvantages in attempting to develop guanethidine as an *in vivo* probe for FMO. Apart from the difficulties in assaying guanethidine and its *N*-oxide (this requires ^{14}C-labelled drug), the drug is incompletely absorbed, and has a half-life of several days. As far as can be ascertained, no reports have appeared to date on the use of guanethidine as an *in vivo* FMO probe.

An ideal *in vivo* FMO probe should be extensively converted by FMO to an enzyme specific metabolite, and both should be readily measurable in blood/plasma and urine. Trimethylamine (TMA) and ethyl methyl sulphide (EMS) are specific FMO substrates (see Section 5), and these compounds were chosen as a starting point for develop-

ment of *in vivo* FMO probes, although it was recognised that perhaps only TMA may be suitable for use in man.

4.3. Modulation of FMO activity

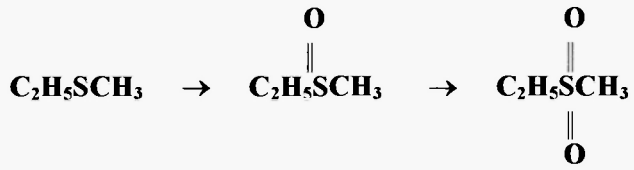
Once a probe has been selected, whereas the relevant pharmacokinetic parameters (e.g. clearance) are readily measured by simple pharmacokinetic studies, it is important to be able to manipulate FMO activity to validate the probe; e.g. induction should result in increased drug clearance and decreased drug plasma half-life, and inhibition should result in the opposite. The use and limitations of methimazole and DMSO for modulation of FMO activity have already been discussed (Section 1.2). Other approaches that could be utilised include: a) Streptozotocin induced diabetes — liver microsomal FMO activity towards thiobenzamide was reported to be increased two-fold in streptozotocin diabetic (insulin deficient) rats and mice /107/; b) induction of FMO by sex steroids and glucocorticoids — modulation of rabbit lung and kidney levels of FMO, as measured by microsomal *N,N*-dimethylaniline *N*-oxygenation, were demonstrated to be induced by one or more of the following: dexamethasone, progesterone, beta-oestradiol, aldesterone, or beta-oestradiol *plus* progesterone following subcutaneous dosing /108/; c) modulation of FMO by ascorbic acid and food restriction — FMO activity towards *N,N*-dimethylaniline and thiobenzamide was reduced significantly in ascorbic acid deficient guinea-pigs /109/, and d) modulation of FMO by feeding orally or parenterally chemically defined diets essentially free from food-derived xenobiotic FMO substrates (see Section 1.2) /19,20/. The last approach, being simple and non-invasive, was chosen in our preliminary studies /20,26,27/ to modulate FMO activity, for the purpose of validating TMA and EMS as potential pharmacokinetic FMO probes (see Section 5).

5. USE OF ETHYL METHYL SULPHIDE (EMS) AND TRIMETHYLAMINE (TMA) AS PHARMACOKINETIC PROBES FOR FMO

5.1. Background and reasons for choosing EMS and TMA

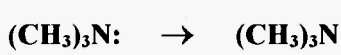
Ethyl methyl sulphide (EMS) is a simple nucleophilic sulphur-containing compound encountered in petroleum distillates and in the

environment. EMS is biotransformed into its corresponding sulphoxide and sulphone derivatives both *in vitro* and *in vivo*.



Two distinct enzymatic pathways seem to be involved in this sulphoxidation process in the rat; it would appear that the flavin-containing monooxygenase is responsible for the *S*-oxidation of EMS to its sulphoxide, while both cytochrome P-450 and FMO are involved in the further oxidation of the sulphoxide to the sulphone /27/. This conclusion was based on comparative studies which examined the effects of various potential metabolic inhibitors, activators, inducing agents, differential heat treatment and chemically defined diets on the rat hepatic microsomal *S*-oxygenation of EMS and *O*-deethylation of 7-ethoxycoumarin (7-EC) /27/. For example, whereas *O*-deethylation of 7-EC was not significantly affected, *S*-oxygenation of EMS decreased by 65% in livers of rats placed on a chemically defined diet for 7 days. The activity returned to normal within 7 days when rats placed on the synthetic diet for 7 days were then allowed free access to normal rat chow /27/.

Trimethylamine (TMA) is a strongly basic simple teriatry aliphatic amine. TMA is a normal constituent of human urine and is derived mainly from common dietary components such as choline and carnitine /103/. TMA undergoes extensive *N*-oxidation *in vivo* to form the polar metabolite trimethylamine *N*-oxide (TMAO) and this reaction is mediated by the flavin-containing monooxygenase *via* an ionic mechanism /86/.



The enzymology of TMA *N*-oxidation was not studied by the present investigators, but the bulk of the literature on TMA suggests that *N*-

oxidation of this substrate is mediated exclusively *via* the FMO (e.g. /86/).

It is notable that TMA or EMS blood concentration data derived from intravenous and oral dosing had not been used until recently to assess the *in vivo* activity of FMO. The blood pharmacokinetic profile of trimethylamine and its *N*-oxide, and EMS and its *S*-oxygenated metabolites, could serve as useful alternative approaches for monitoring the activity and regulation of FMO in live animals, and to probe the genetic disorder, trimethylaminuria (with TMA). The development of EMS and TMA as pharmacokinetic probes for FMO required (a) the characterisation of the pharmacokinetics of EMS and TMA at different doses following intravenous administration and (b) the evaluation of modifications to the pharmacokinetics of EMS and TMA produced by placing male Wistar rats on a chemically defined diet.

5.2. Design of study for validation of probes

Male Wistar rats (230-280 g) were divided into two groups of at least 6 rats each. The control group was maintained on normal rat chow while the other group was placed on a synthetic diet [47% dextrin (type 2), 23% sucrose, 10% corn oil, 14% amino acids and supplemented with vitamins and minerals] for seven days. The rats were surgically prepared under hypnorm®/hypnovel® anaesthesia by implanting indwelling cannulae into the jugular vein and the carotid artery, 24 hours prior to drug administration and blood sampling. EMS and TMA were administered as single bolus doses *via* the jugular vein at 10, 20 and 40 mg/kg dose levels to rats maintained on normal rat chow to establish the linearity of the pharmacokinetics of EMS and TMA. In order to determine the alteration in the pharmacokinetics of EMS and TMA caused by the synthetic diet, single bolus doses were administered through the jugular vein at 20 mg/kg dose to rats in each group. Arterial blood samples (250 μl) were collected in heparinized tubes pre-dose, and post-dose at 5, 15, 30, 45 mins, and at 1, 2, 3, 4, 5, 6, 7 and 8 hours.

Blood levels of TMA and EMS were analysed by sensitive head space gas chromatographic assays /27/. Full details of the experimental design and assay procedures have been reported previously /20,26,27/.

Model-independent pharmacokinetic parameters and significance using Student's paired *t*-test were calculated using the computer software Topfit *v* 1.0 and Minitab, respectively.

5.3. Pharmacokinetics of EMS and TMA in male Wistar rats fed on normal rat chow, or on a synthetic diet

The pharmacokinetics of EMS in control rats were linear over the dose range investigated (AUC_s: 10 mg/kg = 0.54 ± 0.10, 20 mg/kg = 0.93 ± 0.20, and 40 mg/kg = 1.91 ± 0.53 $\mu g.ml^{-1}.h$, r = 0.997). Feeding male Wistar rats with the synthetic diet resulted in a significant (Student's *t*-test) decrease in EMS clearance (53%) and terminal elimination rate (52%). The terminal half-life of EMS was prolonged (46%) and the AUC increased by *c.* two-fold. However the volume of distribution remained unchanged in the two groups /20/ (Table 3).

TABLE 3

Pharmacokinetic parameters of EMS (20 mg/kg, i.v.) in male Wistar rats fed on normal rat chow, or the synthetic diet.

	Normal rat chow	Synthetic diet	Statistical significance (*p* value)
$K_{el}(h^{-1})$	4.90 + 1.01	2.89 ± 0.27	$p < 0.01$
$t_{½}(h)$	0.14 ± 0.03	0.24 ± 0.02	$p < 0.05$
$Cl(ml.min^{-1}.kg^{-1})$	398 + 121	186 ± 21	$p < 0.01$
$Vd(l.kg^{-1})$	4.10 ± 0.69	3.90 ± 0.57	$p > 0.05$
$AUC(\mu g.ml^{-1}.h)$	0.93 ± 0.20	1.81 ± 0.21	$p < 0.05$

n – 6; data from Nnane and Damani /20/.

The pharmacokinetics of TMA were linear up to a dose level of 20 mg/kg (AUC_s: 10 mg/kg = 8.92 ± 0.74, 20 mg/kg = 17.43 ± 4.98, and 40 mg/kg = 26.1 ± 3.45 $\mu g.ml^{-1}.h$). Feeding the rats with the synthetic diet resulted in a significant decrease of TMA clearance (53%) and Vd (50%), and a significant increase in area under the curve (50%). However, the terminal half-life and the terminal elimination rate remained unchanged between the two groups /20/ (Table 4).

The change in Vd for TMA may be due to diet-induced changes in the degree of binding of the probe to plasma constituents. Until clarification is obtained for these data, it is proposed that TMA be used as a probe with caution. The present authors are currently examining TMA pharmacokinetics (and protein binding) in more detail.

TABLE 4

Pharmacokinetic parameters of TMA (20 mg/kg) in male Wistar rats fed on normal rat chow, or the synthetic diet

	Normal rat chow	Synthetic diet	Statistical significance (p value)
$K_{el}(h^{-1})$	0.35 ± 0.09	0.34 ± 0.06	$p > 0.05$
$t_{½}(h)$	2.12 + 0.60	2.11 ± 0.37	$p > 0.05$
$Cl(ml.min^{-1}.kg^{-1})$	20.97 ± 5.92	9.83 ± 1.56	$p < 0.01$
$Vd(l.kg^{-1})$	3.64 + 0.95	1.83 + 0.54	$p < 0.05$
$AUC(\mu g.ml^{-1}.h)$	17.43 ± 4.98	34.65 ± 5.64	$p < 0.01$

n = 6; data from Nnane and Damani /26/.

The results of these first attempts to develop FMO pharmacokinetic probes showed that the *in vivo* disposition of EMS and TMA are altered in male Wistar rats kept on the synthetic diet. The pharmacokinetic parameters of EMS and TMA between the two groups are consistent with a diminished metabolising enzyme activity in rats placed on the synthetic diet. Thus the pharmacokinetic parameters of EMS and TMA, especially the intrinsic clearance, could be useful indices for monitoring the activity of FMO in intact animals, and such simple model compounds may therefore be useful probes for more detailed studies on the regulation of FMO *in vivo*, at least in experimental animals.

6. CONCLUSIONS AND FUTURE PERSPECTIVES

The flavin-containing monooxygenase (FMO) is not inducible by typical cytochrome P-450 inducers such as the barbiturates and polycyclic aromatic hydrocarbons. Furthermore, there are no selective mechanism based inducers or inhibitors of FMO at present. The lack of specific inducers or inhibitors for manipulating FMO activity has hindered studies on the regulation of the activity of this enzyme in intact animals.

Recently, Kaderlik *et al.* /19/ and studies in our laboratory /20,26,27/ have demonstrated the selective nutritional control of liver FMO activity *in vitro*. Chemically defined diets, essentially free from

soft nucleophiles such as plant alkaloids and organo-sulphur compounds which are potential inducers of FMO, resulted in down-regulation of FMO. An attempt was therefore made to develop pharmacokinetic markers for monitoring the activity of FMO in intact animals, with dietary modulation of FMO. The results of these pharmacokinetic studies showed that the *in vivo* disposition of EMS and TMA are altered in male Wistar rats kept on a synthetic amino acid diet. Furthermore, the changes in the pharmacokinetic parameters for EMS and TMA and their *N*- and *S*-oxygenated metabolites /20,26,27/ were consistent with a diminished metabolising enzyme activity in rats placed on the synthetic diet. It would therefore appear that the activity of liver FMO can be modulated by selective nutritional control. Apparently, the flavin-containing monooxygenase may be inducible by soft nucleophiles present in virtually all food obtained from plant sources. It would be interesting to isolate and characterise such inducing agents in animal feed. This would undoubtedly facilitate studies on the regulation of this enzyme in intact mammals.

The use of EMS and TMA as potential pharmacokinetic probes for monitoring the activity of FMO *in vivo* is not without some drawbacks. The sulphoxidation of EMS to EMSO is exclusively an FMO mediated pathway. However, the further *S*-oxidation of EMSO to the corresponding sulphone ($EMSO_2$) involved both cytochrome P-450 and the FMO systems /27/. Obviously, this complicates the kinetic calculations and the conclusions that can be drawn from them. Similarly, the *N*-oxidation of TMA is principally an FMO mediated reaction /86/. However, the spontaneous breakdown of TMAO in rat blood /27/ makes it difficult to assess the kinetics unambiguously. Ideally, pharmacokinetic probes for FMO, and indeed for any enzyme system, should be converted primarily to a single enzyme specific product which does not undergo significant further biotransformation or chemical degradation (see Sections 3.2 and 4.2).

The induction or down-regulation of FMO by components of the human diet could have profound influences on the disposition and biological action of a large number of drugs used in medicine that are substrates for FMO. In fact, drugs from virtually all therapeutic categories are *S*-oxygenated or *N*-oxygenated by FMO. Known substrates for FMO are most prevalent among antipsychotic, antihistamine, anti-rheumatic and narcotic drugs. The effect of modulation of human FMO activity on the disposition and resultant pharmacological

and/or toxicological action of these therapeutic drugs metabolised by FMO could form the basis of a whole new area for future research.

From the data available in the literature, it would appear that FMO activity may be modulated in experimental animals by components in diet, by drug treatment, or by induced diseased states. If these data are valid for man, then it might be expected that FMO is down-regulated in patients on total parenteral nutrition (TPN), or in subjects on faddish diets (e.g. slimming dietary products). Equally, diets deficient in ascorbic acid may lead to a reduction in FMO capacity. On the other hand, again if experimental and animal data can be shown to be valid in man, FMO capacity might be increased in diabetic patients, or in patients undergoing treatment with sex steroids and glucocorticoids. As yet there are no data in the literature for FMO regulation in man, but the data from experimental animals suggest that it is important to develop simple pharmacokinetic probes that might be used to study FMO capacity in humans. Alteration in the activity of human FMO, an important human drug metabolising enzyme, by various factors, as discussed above, could potentially alter the rate of metabolism of a large number of potent pharmaceutical agents, with possible pharmacological and/or toxicological consequences.

As is clear from studies on the assessment of P-450 drug oxidising activity *in vivo*, the multiplicity of the FMO must be taken into account in future studies, since one probe drug is unlikely to differentiate between activities of the different tissue and species-specific FMO isoforms. However, this will need to await the identification of isoform-specific FMO *in vitro* probes from studies with pure mammalian FMO isoforms that are being carried out in several laboratories at present.

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