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Guanethidine *N*-oxidation in human liver microsomes

M. E. McMANUS*, D. S. DAVIES†, A. R. BOOBIS†, P. H. GRANTHAM‡, P. J. WIRTH‡, *Department of Clinical Pharmacology, School of Medicine, Flinders University, Bedford Park 5042, Australia, †Royal Postgraduate Medical School, London W12 0HS, UK, ‡National Cancer Institute, Bethesda, Maryland 20205, USA*

The capacity of human liver microsomes to *N*-oxidize guanethidine from 25 subjects has been assessed. Guanethidine *N*-oxidation was optimal at pH 8·5 and proceeded at only 16% of the maximal rate at pH 7·4. The mean rates of guanethidine *N*-oxidation at pH 8·5 and 7·4 were 2·46 ± 0·89 (mean ± s.d., n = 25) and 0·38 ± 0·22 (mean ± s.d., n = 22), respectively. Interindividual differences in the rate of guanethidine *N*-oxidation at pH 8·5 and 7·4 were 17- and 11-fold, respectively. The cytochrome P450 inhibitors, proadifen and 2,4-dichloro-6-phenylphenoxyethylamine (DPEA), at both pH 8·5 and 7·4 caused less than 20% reduction in the rate of guanethidine *N*-oxidation by human liver microsomes. These data indicate that guanethidine *N*-oxidation can be used as a measure of flavin-containing monooxygenase activity in human liver.

The metabolism of nitrogen- and sulphur-containing xenobiotics has been shown to be carried out by both the flavin-containing and cytochrome P450 monooxygenase enzyme systems (Ziegler 1980; Neal & Halpert 1982). While the cytochrome P450 system in man has been extensively investigated (Boobis & Davies 1984), and nine forms of this enzyme have been purified to electrophoretic homogeneity from human liver (Guengerich et al 1986), the flavin-containing monooxygenase (FCMO) has essentially been neglected (Gold & Ziegler 1973; Rane 1973; Frederick et al 1982; McManus et al 1987). The exact physiological role of the FCMO is unknown. Furthermore, the inherent instability of this enzyme together with the fact that cytochromes P450 also metabolize many of its substrates, have hampered delineating the importance of this enzyme in xenobiotic metabolism. Cysteamine is the only known physiological substrate of the FCMO and is oxidized to its disulphide cystamine (Ziegler & Poulsen 1977). Because the FCMO is capable of oxidizing cysteamine in the presence of a strong reducing environment within the cell, Ziegler & Poulsen (1977) have proposed that its physiological role is in maintaining the cellular thio : disulphide ratio.

The FCMO was initially referred to as the microsomal mixed function amine oxidase but subsequent studies have shown that it catalyses a diverse group of nucleophilic nitrogen and sulphur compounds. These include secondary and tertiary amines, secondary hydroxylamines, hydrazines, thiocarbamides, thioamides, sulphides, disulphides, thiols and sulphenic acids (Ziegler 1980, 1984; Poulsen 1981). A number of medicinal tertiary amines have been shown to be principally metabolized by the FCMO (Ziegler 1980). In a previous study we showed that the *N*-oxidation of the cyclic aliphatic tertiary nitrogen in guanethidine was an FCMO-mediated reaction in rat hepatocytes and microsomes (McManus et al 1983). This substrate besides undergoing *N*-oxidation in man is also metabolized to a ring-opened metabolite, 2-(6-carboxyhexylamino)ethylguanidine (Lukas 1973). In this study we have used guanethidine *N*-oxidation for investigations on the FCMO in human liver microsomes.

Materials and methods

Guanethidine sulphate and 2,4-dichloro-6-phenylphenoxyethylamine (DPEA) were kind gifts of Ciba Pharmaceutical Company, NJ, and Lilly Research Laboratories, IN, USA, respectively. Proadifen was a kind gift of Smith Kline and French Laboratories, PA, USA. [^{14}C]Guanethidine was synthesized according to Abramson et al (1969). NADPH was obtained from Sigma Chemical Company, MO, USA. All other chemicals were of the highest purity commercially available.

Human tissue samples. Microsomal fractions of human liver were obtained as described previously (McManus et al 1984), either from wedge biopsy samples taken at laparotomy or from samples of liver from renal transplant donors maintained on life-support systems until the kidneys could be removed. The use of such tissue in these studies had local Research Ethics Committee permission and, where appropriate, coroner's permission. Samples were stored at −80 °C until required, during which time there was no loss of activity. Liver samples from renal transplant donors had activities very similar to that of wedge biopsy samples with normal histology (Kahn et al 1982) and therefore no further distinction has been made between the 2 groups of samples in this study.

Guanehidine N-*oxidation.* Guanethidine *N*-oxidation was determined by the method of McManus et al (1983). Briefly, a 0·5 mL incubation mixture contained 1 μmol [^{14}C]guanethidine, 0·8 μmol NADPH, 1·0 mg of microsomal protein, 100 μmol potassium phosphate buffered to pH 7·4 or 100 μmol glycine and 25 μmol pyrophosphate buffered to pH 8·4, unless otherwise indicated. Because of the instability of the FCMO in the absence of NADPH, the microsomal protein was always added to the reaction mixture after this coenzyme (Ziegler 1980).

* Correspondence.

NADPH was omitted from the blanks and replaced by an equal volume of buffer. All reactions were commenced by the addition of substrate and carried out in air at 37 °C in a metabolic shaker for 10 min, unless otherwise indicated. Reactions were stopped by the addition of 0·4 mL of acetonitrile and spun at 2500*g* for 10 min to precipitate protein. A 150 μL aliquot was taken for analysis by high performance liquid chromatograpy as previously described (McManus et al 1983). The Michaelis constant (K_m) and the maximum reaction velocity were determined as previously described (McManus et al 1983).

Results

Fig. 1 shows the optimal reaction conditions for guanethidine *N*-oxidation in human liver microsomes. The rate of *N*-oxide formation was linear with time to 30 min and with protein concentration up to 1 mg per 0·5 mL of incubation mixture at both pH 8·5 and 7·4. As observed in other species (Ziegler 1980; Sabourin & Hodgson 1984), *N*-oxidase activity is optimal near pH 8·5 and at pH 7·4 guanethidine *N*-oxidation proceeded at only 16% of the maximal rate (Fig. 2). The Michaelis Menten parameters, K_m and V_{max}, characterizing the reaction in human liver microsomes pooled from six subjects at pH 8·5 and 7·4 were 1·65 ± 0·27 mM, 4·98 ± 0·48 nmol mg^{-1} min^{-1} and 1·63 ± 0·41 mM and 3·9 ± 1·4 nmol mg^{-1} min^{-1}, respectively.

Fig. 3 shows the rate of guanethidine *N*-oxidation in human liver microsomes from 25 subjects. For three subjects it was not possible, owing to the small amount of protein available, to determine their capacity to *N*-oxidize guanethidine at both pH values, consequently for those subjects only data for pH 8·5 is available. The mean rates of guanethidine *N*-oxidation at pH 8·5 and 7·4 were 2·46 ± 0·89 (mean ± s.d., n = 25) and 0·38 ± 0·22 (mean ± s.d., n = 22), respectively. Interindividual differences in the rate of guanethidine *N*-oxidation at pH 8·5 and 7·4 were 17- and 11-fold, respectively. As expected from the pH optima curve above, the rate of guanethidine *N*-oxidation was considerably elevated in all samples tested at pH 8·4 compared with pH 7·4. However, the pH-dependent increase in activity varied markedly between subjects and no statistically significant correlation (r = 0·41; $P > 0·05$) existed between enzyme activity at the two pH values.

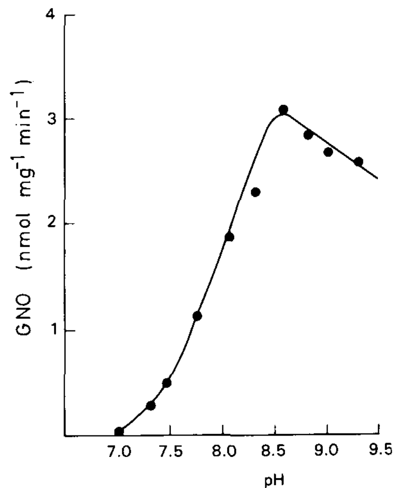

Fig. 2. Effect of pH on guanethidine *N*-oxidation (GNO) in human liver microsomes pooled from four subjects.

Delineating the relative importance of the FCMO in xenobiotic metabolism has been hampered by the fact that many of its substrates are also metabolized by cytochromes P450. Methods used to differentiate between the FCMO and cytochrome P450 enzyme

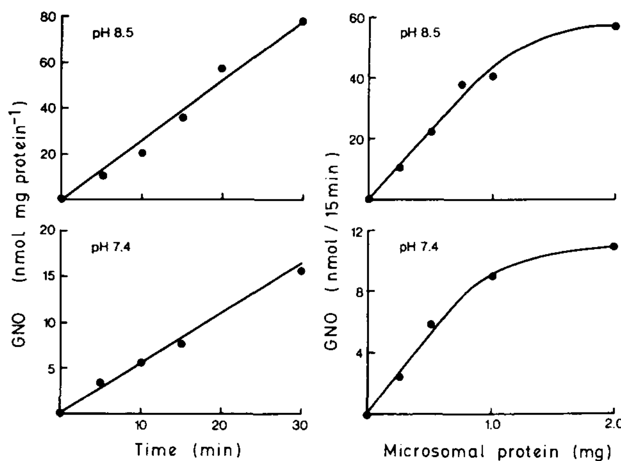

Fig. 1. Effect of protein concentration and time on guanethidine *N*-oxidation (GNO) by human liver microsomes pooled from four subjects at pH 8·5 and 7·4.

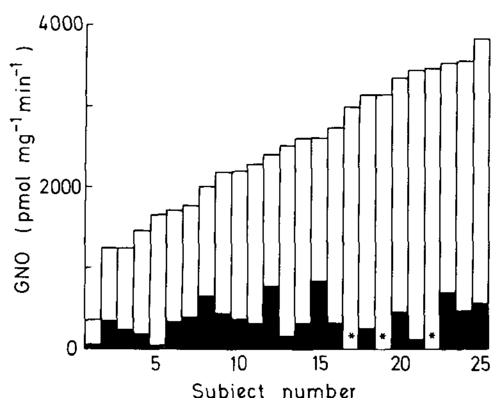

FIG. 3. Guanethidine *N*-oxidation (GNO) in human liver microsomes from 25 subjects at pH 8·5 and 7·4. Asterisk indicates that values were not determined; □ pH 8·5, ■ pH 7·4.

Table 1. Effect of cytochrome P450 inhibitors on human liver microsomal guanethidine *N*-oxide formation.

Addition	Concn (mM)	Guanethidine *N*-oxide formation (nmol mg^{-1} min^{-1}) pH 7·4	pH 8·4
Buffer control	—	0·59	2·12
Proadifen	1·0	0·49	2·24
	2·0	0·49	2·35
DPEA	1·0	0·48	1·89
	2·0	0·49	1·90

Values represent the average of duplicate determinations in microsomes pooled from three subjects. The variability in guanethidine *N*-oxidation within a duplicate pair was less than 5%.

systems have generally employed inhibitors of cytochrome P450 (Gorrod & Damani 1979; Ziegler 1980; Tynes & Hodgson 1983; McManus et al 1983). Table 1 shows the effect of the cytochrome P450 inhibitors, proadifen and DPEA, on guanethidine *N*-oxidation at both pH values. In the presence of these inhibitors a reduction of less than 20% in the rate of *N*-oxide formation at either pH was observed.

Discussion

While the cytochrome P450 monooxygenase system has been extensively investigated both in-vivo and in-vitro in man (Boobis & Davies 1984), the flavin-containing monooxygenase has received only limited attention (Gold & Ziegler 1973; Rane 1973; Frederick et al 1982; McManus et al 1987). In pig liver microsomes the FCMO can comprise up to 4% of microsomal protein (Poulsen & Ziegler 1979) and it appears from in-vitro laboratory animal data that this monooxygenase is the principal enzyme catalysing the oxidation of functional groups bearing nucleophilic nitrogen and sulphur (Ziegler 1980, 1984). Results from the present study show that the cyclic aliphatic tertiary amine guanethidine in human liver microsomes is almost exclusively metabolized by the FCMO at both pH 7·4 and 8·5. This conclusion is based on the fact that guanethidine *N*-oxidation exhibited a pH optimum of 8·5, which is typical of a FCMO-mediated reaction (Ziegler 1980), and the cytochrome P450 inhibitors proadifen and DPEA caused less than a 20% decrease in the rate of this reaction. The primary amine DPEA besides being a cytochrome P450 inhibitor has also been shown to increase FCMO activity by binding to an effector site (Ziegler 1980; McManus et al 1983, 1987). In a previous study we found that DPEA increased the rate of dimethylaniline *N*-oxidation in three out of four human liver microsomes tested (McManus et al 1987). These data indicate that stimulation of FCMO activity by primary amines can depend both on the substrate and source of human tissue used.

Similar to in-vitro cytochrome P450 studies utilizing human liver microsomes (Boobis & Davies 1984; McManus et al 1984), marked inter-individual differences in the rate of guanethidine *N*-oxidation (FCMO activity) was observed between subjects. The lack of a statistically significant correlation between guanethidine *N*-oxidation at pH 8·5 and 7·4 was unexpected considering the K_ms for the reaction at both pH values were identical. Recently both Williams et al (1985) and Tynes & Hodgson (1985) have purified the rabbit lung FCMO and have shown this enzyme to be immunochemically and catalytically distinct from the form present in rabbit liver. These studies are the first indication that the FCMO can exist as more than a single form. The lack of a correlation between the rates of guanethidine *N*-oxidation at pH 8·5 and 7·4 may reflect the differential effect of pH on different isozymes of the FCMO.

In summary, the results of the present study demonstrate that guanethidine *N*-oxidation appears to be a good probe for the flavin-containing monooxygenase in human liver microsomes. In a previous study, McMartin & Simpson (1971) have shown that the *N*-oxide of guanethidine can account for as much as 50% of the excreted dose of this amine. These data taken together clearly indicate the utility of guanethidine as a metabolic probe for studying the flavin-containing monooxygenase enzyme in-vivo in man.

REFERENCES

Abramson, F. B., Furst, C. I., McMartin, C., Wade, R. (1969) Biochem. J. 113: 143–156

Boobis, A. R., Davies, D. S. (1984) Xenobiotica 14: 151–185

Frederick, C. B., Lays, J. B., Ziegler, D. M., Guengerich, F. P., Kadlubar, F. F. (1982) Cancer Res. 42: 2671–2677

Gold, M. S., Ziegler, D. M. (1973) Xenobiotica 3: 179–189

Gorrod, J. W., Damani, L. A. (1979) Ibid. 9: 219–226

Guengerich, F. P., Distlerath, L. M., Reilly, P. E. B., Wolff, T., Shimada, T., Umbenhauer, D. R., Martin, M. V. (1986) Ibid. 16: 367–378

Kahn, C. C., Boobis, A. R., Murray, S., Brodie, M. J., Davies, D. S. (1982) Br. J. Clin. Pharmacol. 13: 637–645

Lukas, G. (1973) Drug Metab. Rev. 2: 101–116

McManus, M. E., Grantham, P. H., Cone, J. L., Roller, P. P., Wirth, P. J., Thorgeirsson, S. S. (1983) Biochem. Biophys. Res. Commun. 112: 437–443

McManus, M. E., Boobis, A. R., Minchin, R. F., Schwartz, D. M., Murray, S., Davies, D. S., Thorgeirsson, S. S. (1984) Cancer Res. 44: 5692–5697

McManus, M. E., Stupans, I., Burgess, W., Koenig, J. A., Hall, P. de la M., Birkett, D. J. (1987) Drug Metab. Dispos. 15, 256–261

McMartin, C., Simpson, P. (1971) Clin. Pharmacol. Ther. 12: 73–77

Neal, R. A., Halpert, J. (1982) Ann. Rev. Pharmacol. Toxicol. 22: 321–339

Poulsen, L. L. (1981) Rev. Biochem. Toxicol. 3: 33–49

Poulsen, L. L., Ziegler, D. M. (1979) J. Biol. Chem. 254: 6449–6455

Rane, A. (1973) Clin. Pharmacol. Ther. 15: 32–88

Sabourin, P. J., Hodgson, E. (1984) Chem.-Biol. Interact. 51: 125–139

Tynes, R. E., Hodgson, E. (1983) Biochem. Pharmacol. 32: 3419–3428

Tynes, R. E., Hodgson, E. (1985) Arch. Biochem. Biophys. 240: 77–93

Williams, D. E., Hale, S. E., Muerhoff, S. S., Masters, B. S. S. (1985) Mol. Pharmacol. 28: 381–390

Ziegler, D. M. (1980) in: Jakoby, W. B. (ed.) Enzymatic Basis of Detoxication, Academic Press, New York, pp 201–227

Ziegler, D. M. (1984) in: Mitchell, J. R., Horning, M. G. (eds) Drug Metabolism and Drug Toxicity, Raven Press, New York, pp 33–53

Ziegler, D. M., Poulson, L. L. (1977) Trends Biochem. Sci. 2: 79–81

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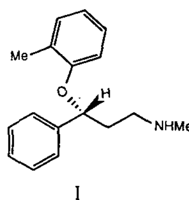
Letter to the Editor

Tomoxetine and the stereoselectivity of drug action

ROBERT OBERLENDER, DAVID E. NICHOLS*, P. V. RAMACHANDRAN†, MORRIS SREBNIK†, *H. C. Brown & R. B. Wetherill Dept of Medicinal Chemistry and Pharmacognosy, School of Pharmacy and Pharmacal Sciences, and †Laboratories of Chemistry, Purdue University, West Lafayette, Indiana 47907, USA*

The phenoxypropylamine derivative tomoxetine has been shown to inhibit noradrenaline (NA) uptake into synaptosomes from rat hypothalamus (Wong et al 1982). Differences in the potencies of the resolved optical isomers, (−)-tomoxetine (I) and (+)-tomoxetine were also noted, the (−)-isomer being more potent than the racemate or the (+)-isomer in-vitro and in-vivo.

Our attention was caught by the use of the term 'stereoselectivity' in the discussion of NA uptake inhibition by Wong et al (1982). The relative potencies of the (+)- and (−)-isomers of tomoxetine and amphetamine (IV is the more potent (+)-isomer of amphetamine) shown were compared, even though the asymmetric centres for these molecules are not similar. In addition, the stereoselectivity of tomoxetine and NA (III is the biologically relevant (−)-isomer of NA) was discussed with reference to their signs of optical rotation, without regard to absolute configuration. Finally, it was stated that the stereoselectivity of nisoxetine (II is the more potent (+)-isomer), a close analogue of tomoxetine in which an *ortho*-methoxy group replaces the *ortho*-methyl group in the latter, was reversed relative to tomoxetine. If both compounds interact with the same acceptor sites in noradrenergic nerve terminals, which seems likely from their similarity



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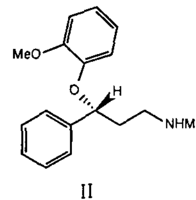

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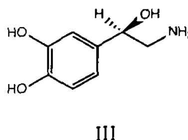

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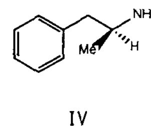

IV

* Correspondence.