Gibaldi, M., Perrier, D. (1982) Pharmacokinetics. 2nd. Ed., Marcel Dekker, New York

Gugler, R., Jensen, J. C. (1984) Lancet i: 969

Hajek, K. K., Cook, N. I., Novak, R. F. (1982) J. Pharmacol. Exp. Ther. 223: 97–104

Igari, Y., Sugiyama, Y., Sawada, Y., Iga, T., Hanano, M. (1982) Drug Metab. Disp. 10: 676–679

Igari, Y., Sugiyama, Y., Sawada, Y., Iga, T., Hanano, M. (1985) Ibid. 13: 102–106

Kapetanovic, I. M., Kuppferberg, H. J. (1984) Ibid. 12: 560–563

Klotz, U., Reimann, I. (1980) Eur. J. Clin. Pharmacol. 18: 517–520

Klotz, U., Reimann, I. (1981) Clin. Pharmacol. Ther. 30: 513–517

Klotz, U., Avant, G. R., Hoyumpa, A., Schenker, S., Wilkinson, G. R. (1975) J. Clin. Invest. 55: 347–359

Klotz, U., Antonin, K.-H., Bleck, P. R. (1976) J. Pharmacol. Exp. Ther. 199: 67–73

Klotz, U., Antonin, K. H., Brugel, H., Bleck, P. R. (1977) Clin. Pharmacol. Ther. 21: 430–436

Klotz, U., Anttila, V.-J., Riemann, I. (1979) Lancet ii: 699

Mennini, T., Gobbi, M., Testa, R. (1984) Life Sci. 35: 1811–1820

Meredith, C. G., Maldonado, A. L., Speeg, K. V. Jr. (1985) Drug Metab. Disp. 13: 156–162

Nardi, D., Tajana, A., Leonardi, A., Pennini, R., Portioli, F., Magistretti, M. J., Subissi, A. (1981) J. Med. Chem. 24: 727–731

Puurunen, J., Pelkonen, O. (1979) Eur. J. Pharmacol. 55: 335–336

Randall, L. O., Kappell, B. (1973) in: Garattini, S., Mussini, E., Randall, L. O. (eds) The Benzodiazepines. Raven Press, New York, pp 27–51

Rendic, S., Sunjic, V., Toso, R., Kajfez, F., Ruf, H.-H. (1979) Xenobiotica 9: 555–564

Sacchi Landriani, G., Guardabasso, V., Rocchetti, M. (1983) Comput. Programs Biomed. 16: 35–42

Schwartz, M. A., Koechlin, B. A., Postma, E., Palmer, S., Krol, G. (1965) J. Pharmacol. Exp. Ther. 149: 423–435

J. Pharm. Pharmacol. 1986, 38: 472–475
Communicated November 25, 1985

Inhibition of hepatic microsomal monooxygenase activity by cinchocaine: mechanistic studies and effects of ionization

MICHAEL MURRAY, *Department of Medicine, University of Sydney, Westmead Centre, Westmead, NSW 2145, Australia*

The quinoline-based local anaesthetic cinchocaine (dibucaine) was found to be a mixed-type inhibitor of microsomal aminopyrine *N*-demethylase and 7-ethoxycoumarin *O*-deethylase activities from control and phenobarbitone-induced rat liver in-vitro. Cinchocaine also elicited a characteristic type I optical difference spectrum in oxidized liver microsomes (K_s = 24 μM; ΔA_{max} = 3·4 × 10^{-3} absorbance units (nmol cytochrome P450)$^{-1}$) but did not appear to bind to the reduced form of the cytochrome. Additional studies indicated that cinchocaine competitively inhibited the type I spectral binding of substrate (aminopyrine) to ferric cytochrome P450. Studies of monooxygenase inhibition by cinchocaine over a relatively narrow pH range (6·5–8·5) indicated that, as might be expected, the un-ionized form of the drug is associated with inhibitory potency superior to that of the ionized form. Thus 40% inhibition of aminopyrine *N*-demethylase activity was observed with 100 μM cinchocaine at pH 8·0 and 8·5 (24% and 50% un-ionized drug, respectively), whereas only 16% inhibition was observed at pH 6·5 (1% un-ionized drug). These findings suggest that the inhibitory action of cinchocaine is mediated exclusively via an interaction with ferric cytochrome P450 and that the extent of ionization is a determinant of mixed function oxidase inhibition.

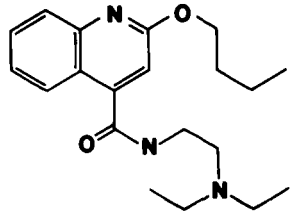
A number of studies have demonstrated that nitrogen heterocycles, including imidazoles (Wilkinson et al 1972, 1974a; Murray & Wilkinson 1984), benzimidazoles (Murray et al 1982; Dickins & Bridges 1982; Murray & Ryan 1983) and quinolines (Kahl & Netter 1977; Back et al 1983; Murray 1984), can alter hepatic microsomal xenobiotic metabolism by inhibiting cytochrome P450 (P450)-dependent mixed-function oxidase (MFO) activity. The nature of spectrally observed interactions between inhibitory xenobiotics and P450 has been used as an indication of the mode of MFO inhibition. For example, monoheterocyclic imidazoles generally elicit type II optical difference spectra with ferric P450, a finding that is consistent with a ligand interaction at the haem iron site (Wilkinson et al 1974a, b). On the other hand, fused-ring benzimidazoles substituted in the 2-position with large alkyl or arylalkyl groupings elicit the type I spectral change (Dickins & Bridges 1982; Murray & Ryan 1983). This finding implies that an interaction occurs between the inhibitor and a hydrophobic patch on the P450 apoprotein (Al-Gailany et al 1978). From Hansch analysis the principal determinant of MFO inhibitor potency, in the case of imidazoles and benzimidazoles, appears to be hydrophobicity (Wilkinson et al 1974a; Murray et al 1982), although the importance of steric effects and the position of heteroaromatic substitution has been stressed (Rogerson et al 1977; Murray & Wilkinson 1984).

Hydrophobic interactions have also been hypothesized to be important in the interaction of local anaesthetics with phospholipid membrane bilayers (Papahadjopoulos 1972). Cinchocaine (dibucaine, I) is a potent local anaesthetic of the amide type that is used typically in ointments or, as the hydrochloride, in infiltration anaesthesia (Martindale 1982). The

presence of the quinoline nucleus in the structure of cinchocaine suggested that this agent may be an effective MFO inhibitor.

As the physicochemical requirements for MFO inhibition by drugs are similar to those for effective local anaesthetic action the present study was undertaken to assess the capacity of cinchocaine to modify rat microsomal aminopyrine *N*-demethylase (APDM) and 7-ethoxycoumarin *O*-deethylase (ECOD) activities from control and phenobarbitone-induced rat liver. This study also evaluated the mechanism of APDM inhibition produced by cinchocaine, and the effect of its ionization on inhibition and binding to oxidized microsomal cytochrome P450.

(I)



Materials and methods

Chemicals. Cinchocaine hydrochloride and all biochemicals were obtained from Sigma Chemical Co., St Louis, MO, USA. All other solvents and reagents were of analytical reagent grade.

Animals. Male Wistar rats (250–300 g) were from the Institute of Clinical Pathology and Medical Research at the Westmead Centre. Rat hepatic microsomes were prepared from untreated or phenobarbitone-induced animals as described by Murray et al (1983). Microsomes were stored as frozen pellets at −20 °C until required for use.

Mixed-function oxidase activities. APDM activity in rat hepatic microsomal fractions was determined as described by Murray et al (1982), except that isocitrate dehydrogenase (1 unit), isocitric acid (5 mM final concentration) and NADP (0·4 mM final concentration) was used as the NADPH-generating system. Incubation pH was varied between 6·5 and 8·5 as dictated by the experimental requirements: NADPH (1 mM) was used in these experiments to obviate any pH effects on the generating system. ECOD activity was determined by the spectrofluorometric method of Prough et al (1978).

Binding studies. Microsomal pellets were resuspended in 0·1 M potassium phosphate buffer containing 1 mM EDTA (pH 7·4) to a protein concentration of 2 mg ml^{-1}. The suspension was then divided equally between two cuvettes and a baseline of zero light absorbance was established in an Aminco DW-2a spectrophotometer operating in the split beam mode. Type I optical difference spectra elicited by aminopyrine and cinchocaine in phenobarbitone-induced microsomes were obtained as described elsewhere (Murray & Ryan 1983). Apparent spectral dissociation constants (K_s) and maximal absorbance changes (ΔA_{max}) were determined from the x- and y-axis intercepts, respectively, of double reciprocal plots of ΔA (peak to trough) vs aminopyrine concentration (four to six different concentrations). In other experiments, the binding charcteristics of the aminopyrine type I interaction in phenobarbitone-induced microsomes were determined in the presence of 0·11 mM cinchocaine. Here, cinchocaine was added in 5 µl of absolute alcohol to both the sample and reference cuvettes and a baseline was established. The aminopyrine type I spectrum was then generated after the introduction of microlitre quantities of a stock solution of aminopyrine (in 0·1 M potassium phosphate buffer, pH 7·4) to the sample cuvette.

The extent of cinchocaine binding in reduced microsomes was evaluated after the addition of NADPH (0·6 M final concentration) or a few crystals of sodium dithionite.

Cytochrome P450 was measured according to the method of Omura & Sato (1964) and microsomal protein content was determined by the method of Lowry et al (1951).

Results and discussion

Under standard incubation conditions (pH 7·4), cinchocaine was found to be a moderate inhibitor of APDM activity from phenobarbitone-induced rat liver microsomes (about 30% inhibition at 100 µM cinchocaine). Kinetic analysis over a range of substrate concentrations revealed that cinchocaine could be characterized as a mixed-type inhibitor with an inhibitor equilibrium constant (K_i) of 0·11 mM (Fig. 1). Similar findings were

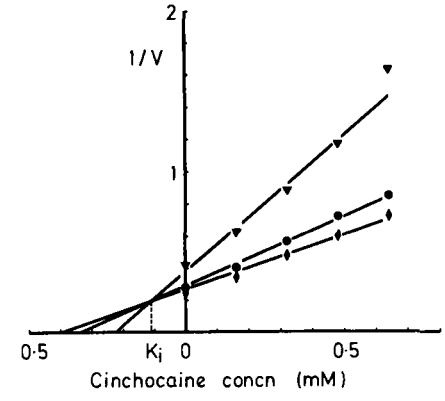


FIG. 1. Dixon plot of inhibitor (cinchocaine) concentration versus the reciprocal APDM activity (1/V where V = nmol (mg protein)$^{-1}$ min^{-1}) at different substrate concentrations; 0·25 (▼), 0·50 (●) and 0·75 mM (◆).

Table 1. K_i values for the inhibition of different microsomal mixed-function oxidase activities by cinchocaine.

Microsomal type	K_i (mM)* Aminopyrine *N*-demethylase	7-Ethoxycoumarin *O*-deethylase
Control	0·24	0·070
Phenobarbitone-induced	0·11	0·13

* K_i values were estimated from Dixon plots of inhibitory data at several substrate concentrations as shown, for example, in Fig. 1.

obtained when cinchocaine was evaluated as an inhibitor of other MFO activities. Thus the K_i value for cinchocaine as an inhibitor of APDM from control liver microsomes was 0·24 mM and the K_i values against ECOD activity from control and phenobarbitone-pretreated microsomes were 0·070 and 0·13 mM, respectively (Table 1).

Cinchocaine was found to elicit a type I optical difference spectrum (λ_{max} = 386 nm; λ_{min} = 422 nm) in oxidized microsomes from phenobarbitone-induced rat liver (not shown). A value of 24 μM was obtained for the spectral dissociation constant (K_s) of the cinchocaine type I interaction indicating a quite high affinity for binding of the drug to ferric P450. However the apparent maximal spectral change (ΔA_{max}) was determined to be $3{\cdot}4 \times 10^{-3}$ absorbance units per nmol P450 which is indicative of a low capacity interaction. Indeed, if the extinction coefficient (ε = 110 mM^{-1} cm^{-1}) reported by Estabrook & Werringloer (1978) is applied, then it is apparent that only about 3% of the total P450 in phenobarbitone-induced rat liver microsomes could be involved in the interaction with cinchocaine. By comparison, the substrate aminopyrine elicited a type I spectral change in these microsomal fractions with K_s = 220 μM and $\Delta A_{max} = 9{\cdot}9 \times 10^{-3}$ absorbance units per nmol P450. Again, applying the literature extinction coefficient indicates that a maximum of approximately 9% of the total P450 may be converted from a low to high spin configuration by aminopyrine. Thus it would appear that a greater proportion of the microsomal P450 is available to undergo a type I interaction with aminopyrine than is available for cinchocaine binding. Nevertheless, studies of aminopyrine type I binding in the presence of cinchocaine demonstrated the capacity of the inhibitor to influence the interaction between P450 and substrate. The inclusion of 0·11 mM cinchocaine increased the apparent K_s of the aminopyrine type I change to 520 μM without affecting the capacity (ΔA_{max}) of the binding interaction. This situation is analogous to competitive inhibition in a catalytic sense, and suggests that cinchocaine and aminopyrine may compete to some extent for the same binding site(s) within the P450 catalytic centre.

Additional studies demonstrated that cinchocaine elicited no optical difference spectrum in either NADPH- or sodium dithionite-reduced liver microsomes (not shown). Thus it must exert its inhibitory effect on APDM activity via an interaction with ferric P450 as it possesses no affinity for reduced P450. It could therefore be concluded that cinchocaine exerts a competitive effect on substrate binding to a portion of the ferric P450 available for enzyme-substrate complex formation. No doubt the observed extent of inhibition reflects the capacity of cinchocaine to influence aminopyrine binding to ferric P450 before NADPH reduction.

pH optima for MFO reactions are usually observed in the 7·0–8·0 range. In the present study both low (6·5) and, in particular, high incubation pH (8·5) were associated with sub-optimal APDM activity (Fig. 2). However, it is apparent from Fig. 2 that this MFO activity is more susceptible to inhibition by cinchocaine at high pH (8–8·5). The pK of the side chain diethylaminoethyl group has been reported to be 8·5 (Singer 1974), whereas the quinoline nitrogen atom is only protonated below pH 4·0 (Moore & Hemmens 1982).

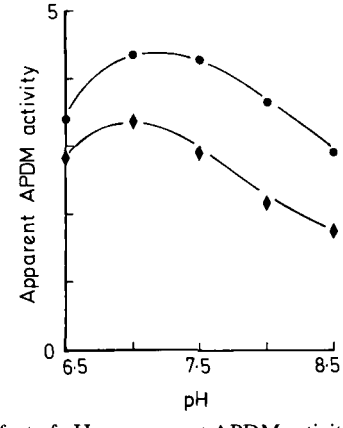


FIG. 2. Effect of pH on apparent APDM activity (nmol (mg protein)$^{-1}$ min^{-1}) in the presence (◆) and absence (●) of 100 μM cinchocaine.

Thus, under standard incubation pH conditions, only 5–10% of the cinchocaine would be present in its un-ionized form. In view of the established importance of lipophilicity to MFO inhibition potency it is probable that extensive ionization of the antagonist would be associated with diminished inhibition. From Fig. 3 it is apparent that the percent inhibition of APDM activity produced by 100 μM cinchocaine increases with pH, as does the fraction of it present in the un-ionized form. Further studies, in which the effect of pH on the affinity and capacity of the cinchocaine type I binding interaction in oxidized microsomes, were also conducted. At low pH (6·5) the K_s of the binding interaction was 36 μM and its ΔA_{max} was $2{\cdot}8 \times 10^{-3}$ absorbance units per nmol P450. In contrast, at pH 8·5, the affinity of cinchocaine for P450 was increased (K_s = 20 μM) as was the maximal

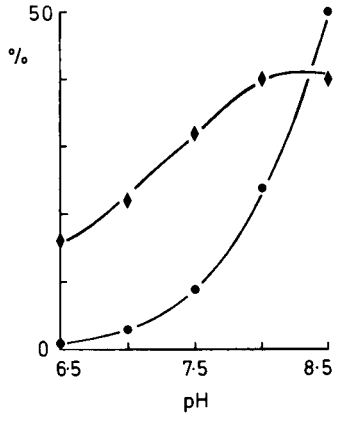


FIG. 3. Effect of pH on percent APDM inhibition produced by cinchocaine (◆) and on the percentage of un-ionized inhibitor (●).

binding capacity ($\Delta A_{max} = 4{\cdot}1 \times 10^{-3}$ absorbance units per nmol P450). These binding data reflect the greater capacity and affinity that P450 has for cinchocaine at elevated pH and are consistent with the superior inhibition of APDM produced by cinchocaine at slightly basic pH. However it is clear that ionized cinchocaine (side-chain diethylaminoethyl group protonated at low pH) retains a degree of affinity for the substrate binding site. This situation is compatible with MFO inhibition if it is proposed that the cinchocaine 4-substituent is projected away from the lipophilic binding region of P450 and into the aqueous medium. From this proposal it seems more likely that the 2-n-butoxy substituent, and not the 4-substituent, is involved in a hydrophobic interaction at the P450 active site. Certainly, if the 4-substituent was involved in an inhibitory binding interaction a more pronounced effect of pH on inhibition may be anticipated.

From the present study it is clear that cinchocaine is capable of influencing microsomal MFO activity (as APDM and ECOD), in both control and phenobarbitone-induced liver fractions, largely via an interaction with oxidized P450. The effect of pH on MFO inhibition may prove important in structure-activity studies with certain classes of antagonists unless the extent of ionization is considered. In addition, it should not be assumed that ionization of the inhibitor removes all MFO antagonist potency.

REFERENCES

Al-Gailany, K. A. S., Houston, J. B., Bridges, J. W. (1978) Biochem. Pharmacol. 27: 783–788

Back, D. J., Purba, H. S., Staiger, C., Orme, M. L., Breckenidge, A. M. (1983) Ibid. 32: 257–263

Dickins, M., Bridges, J. W. (1982) Ibid. 31: 1315–1320

Estabrook, R. W., Werringloer, J. (1978) Methods Enzymol. 52C: 212–220

Kahl, R., Netter, K. J. (1977) Toxicol. Appl. Pharmacol. 40: 473–483

Lowry, O. H., Rosebrough, N. J., Farr, A. L., Randall, R. J. (1951) J. Biol. Chem. 193: 263–275

Martindale (1982) The Extra Pharmacopoeia. 28th edn, The Pharmaceutical Press, London, pp 913–914

Moore, D. E., Hemmens, V. J. (1982) Photochem. Photobiol. 36: 71–77

Murray, M. (1984) Biochem. Pharmacol. 33: 3277–3281

Murray, M., Ryan, A. J. (1983) Xenobiotica 13: 707–714

Murray, M., Wilkinson, C. F. (1984) Chem.-Biol. Interact. 50: 267–275

Murray, M., Ryan, A. J., Little, P. J. (1982) J. Med. Chem. 25: 887–892

Murray, M., Wilkinson, C. F., Dube, C. E. (1983) Toxicol. Appl. Pharmacol. 68: 66–76

Omura, T., Sato, R. (1964) J. Biol. Chem. 239: 2370–2378

Papahajopoulos, D. (1972) Biochim. Biophys. Acta 265: 169–186

Prough, R. A., Burke, M. D., Mayer, R. T. (1978) Methods Enzymol. 52C: 372–377

Rogerson, T. D., Wilkinson, C. F., Hetnarksi, K. (1977) Biochem. Pharmacol. 26: 1039–1042

Singer, M. A. (1974) Ibid. 23: 2939–2950

Wilkinson, C. F., Hetnarksi, K., Yellin, T. O. (1972) Ibid. 21: 3187–3192

Wilkinson, C. F., Hetnarski, K., Cantwell, G. P., Di Carlo, F. J. (1974a) Ibid. 23: 2377–2386

Wilkinson, C. F., Hetnarksi, K., Hicks, L. J. (1974b) Pestic. Biochem. Phyiol. 4: 299–312