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# HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC ASSAY FOR MEXILETINE HYDROXYLATION IN MICROSOMES OF HUMAN LIVER

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## SUMMARY

A simple high-performance liquid chromatographic assay, using fluorescence detection, is described for determining simultaneously the production of the two major hydroxylated metabolites of mexiletine in human liver microsomes. The detection limits of hydroxymethylmexiletine and *p*-hydroxymexiletine are 0.35 and 0.08 nmol/ml, respectively. The assay is specific, reproducible and allows the simultaneous kinetic characterization of the reactions in small amounts of liver tissue. The assay may be used to acquire a better knowledge of the kinetic behaviour of mexiletine and of its metabolites, and to investigate if the large inter-individual variations of the mexiletine pharmacokinetics are of metabolic origin, due to variations of its hydroxylation processes.

## INTRODUCTION

Mexiletine [1-(2′,6′-dimethylphenoxy)-2-aminopropane] (I) is a type 1B antiarrhythmic drug (Fig. 1). Its structure and electrophysiological properties are similar to those of lignocaine. However, mexiletine has a longer half-life (10 h) and is also active by oral administration, owing to its high bioavailability (80–88%). These characteristics make the long-term treatment and the prevention of ventricular arrhythmias possible with this drug [1–3].

The therapeutic index of mexiletine is narrow (0.75–2.0 μg/ml) and a clear relationship exists between the plasma concentration of the drug and both its

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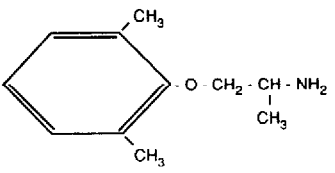
therapeutic and toxic effects [4–7]. Numerous studies [1, 2, 5–9] have shown that, regardless of the route of administration [intravenously (i.v.) or oral (p.o.)], there are important variations of the mexiletine plasma kinetics in patients after a given dose. In these conditions, the relationship between the dose and the plasma concentration of the drug is not easily predictable in an individual and, in some cases, an adjustment of the dose is required to ensure efficacy and to reduce the side-effects of the drug [4]. These variations may have many origins but one of principal factors most likely to influence the plasma concentrations of mexiletine is the capacity of the liver to metabolize the drug [2,10].

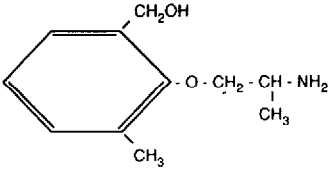
Mexiletine, which has been reported to undergo negligible presystemic elimination [11], is extensively metabolized in man, primarily by hydroxylation in the liver [1,12,13]. Less than 10% of mexiletine is recovered unchanged in urines over 48 h [3,10]. The two major reported metabolites of mexiletine are hydroxymethylmexiletine (II) and *p*-hydroxymexiletine (III) (Fig. 1), which are subsequently eliminated by glucuronide conjugation [3,10,13]. Thus, inter-individual variations in mexiletine pharmacokinetics might originate from variations in mexiletine hydroxylation rates.

Most drug hydroxylation reactions are catalysed by the hepatic mixed function oxidase system, which may be studied to advantage in liver microsomal fractions. Some reports [10,11,14] suggest that the formation of the two major hydroxylated metabolites of mexiletine are mediated by this enzymatic system and probably by two different populations of cytochrome P-450.

In order to find out if the variation in the pharmacokinetics of mexiletine among patients were of metabolic origin and due to variations of mexiletine hydroxylation processes, we have developed a high-performance liquid chromatographic




I

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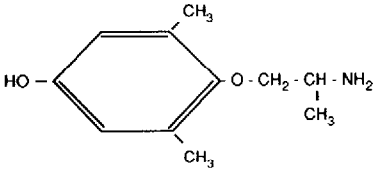


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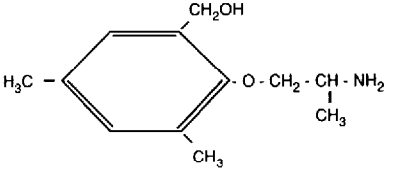


IV

Fig. 1. Structures of mexiletine, its metabolites and the internal standard. I = Mexiletine [1-(2′,6′-dimethylphenoxy)-2-aminopropane]; II = hydroxymethylmexiletine [1-(2′-hydroxymethyl-6′-methylphenoxy)-2-aminopropane]; III = *p*-hydroxymexiletine [1-(4′-hydroxy-2′,6′-dimethylphenoxy)-2-aminopropane]; IV = internal standard [1-(2′-hydroxymethyl-4′,6′-dimethylphenoxy)-2-aminopropane].

(HPLC) assay with fluorescence detection to characterize mexiletine hydroxylation in human liver microsomes.

## EXPERIMENTAL

### *Materials*

Mexiletine (hydrochloride) (I), the synthetic references of its metabolites II (Kö 2259, oxalate) and III (Kö 2127, hydrochloride) and the internal standard (IV) (Kö 5271, oxalate) were generously provided by Boehringer Ingelheim (Reims, France) (Fig. 1). β-Nicotinamide adenine dinucleotide phosphate (sodium salt) (NADP), DL-isocitrate (trisodium salt), isocitrate dehydrogenase (type 4) and bovine serum albumin (fraction V) were supplied by Sigma (St. Louis, MO, U.S.A.). All other chemicals were of analytical-reagent grade.

### *Tissue samples*

Histologically normal human liver tissue was obtained from a patient shortly after circulatory arrest. The liver was cut into small pieces, immediately frozen in liquid nitrogen and stored at −80 °C. Liver microsomes were prepared according to a modification of the method of Boobis et al. [15]. Liver samples (0.2–1 g) were homogenized three times for 30 s in ice-cold buffer [0.1 *M* $KH_2PO_4$–1 m*M* EDTA–0.1 m*M* DL-dithiothreitol (pH 7.4)] using an Ultra-Turrax homogenizer set at 5000 rpm. All manipulations were performed in a cold chamber at 4 °C. Post-mitochondrial supernatant was prepared by centrifugation of the homogenate at 10 500 *g* (TDX 9527-16 centrifuge; Abbot, Rungis, France) for 1 and 8 min, and the supernatant was again centrifuged at the same speed for 10 min. Microsomes were sedimented by ultracentrifugation three times for 60 min at 103 000 *g* (L8-55M centrifuge; SW 60 TI rotor; Beckman, Gagny, France). Between all centrifuge steps, the pellets were washed with 0.1 *M* pyrophosphate buffer (pH 7.4) to remove non-membranous proteins, such as haemoglobin or drugs ingested by patients. The pellets were finally resuspended in 0.1 *M* potassium phosphate buffer (pH 7.4) containing 20% glycerol, and the microsomal suspensions were immediately analysed or frozen in liquid nitrogen and stored at −80 °C. Proteins were measured by a modification of the method of Lowry et al. [16] using crystalline bovine serum albumin fraction V as standard. The assays were perfomed on 10-μl aliquots of microsomal suspensions and ten-fold diluted homogenates.

In order to assume that microsomal suspensions reflect the drug metabolizing capacity of non-diseased livers, they were previously characterized by electron microscopic studies and by the measurement of the specific activity of enzyme markers, cytochrome $b_5$ and cytochrome P-450 (unpublished results).

### *Incubation conditions and extraction procedure*

The incubation mixture consisted of the substrate I (330 μ*M* in maximum velocity studies, 20–400 μ*M* in full kinetic studies) and the NADPH-generating system (4 m*M* magnesium chloride–0.85 m*M* NADP–4.25 m*M* isocitrate–0.85

I.U./ml isocitrate dehydrogenase). All reagents were dissolved in 0.1 *M* potassium phosphate buffer (pH 7.4). After preincubation for 15 min, the reaction was started by addition of 70–600 μg of microsomal proteins (previously kept on ice) to a 300-μl final volume. In blank incubations used as controls, NADPH-generating system, mexiletine or microsomal fractions were replaced with a corresponding volume of incubation buffer. Incubations were performed in glass-capped tubes at 37°C in a shaking water-bath for 4 h. The reaction was stopped by adjusting the milieu to pH 12 with 100 μl of 1 *M* sodium hydroxide solution. Immediately, 100 μl of a 5 μg/ml internal standard solution in potassium phosphate buffer (pH 7.4) were added to each sample. The contents were mixed and extracted with 7 ml of ethyl acetate. After centrifugation at 3000 *g* for 5 min, the organic layer was transferred to another tube and evaporated to dryness under nitrogen at 45°C. Just before analysis, the residue was dissolved in 100 μl of the HPLC mobile phase.

*High-performance liquid chromatographic analysis*

II and III produced in vitro by microsomal fractions were determined by HPLC with fluorescence detection. The samples were analysed using an HP 1090 liquid chromatograph equipped with an automatic injector, connected to an HP 85b computer (Hewlett-Packard, Orsay, France), a T5C recorder (Ifelac, Courbevoie, France) and an RF 530 fluorescence HPLC monitor (Shimadzu, Kyoto, Japan). Separation was performed on a reversed-phase XL 3-μm ODS column (75 mm×4.6 mm I.D.) (Beckman) using a mobile phase consisting of 10 m*M* potassium phosphate buffer (pH 4.2)–methanol–acetonitrile (90:5:5, v/v) and a column temperature of 40°C. The flow-rate was 0.75 ml/min from 0 to 7 min, increased slowly to 1.5 ml/min at 9 min then decreased from 20 min to 0.75 ml/min at 22 min. The detector was set at 270 and 312 nm for the excitation and emission wavelength, respectively, with a recorder "span" of 10 mV full-scale Samples of 25 μl were injected on the column.

Calibration standards were prepared before each study at an appropriate dilution to deliver between 0.6 and 6 nmol/ml II and between 0.3 and 3 nmol/ml III in a 300-μl final volume of incubation buffer. These standards were incubated and extracted under the same conditions as described for the samples. Peak-height ratios of II and III to IV (internal standard) were measured, and calibration graphs were obtained from linear regression analyses of the peak-height ratios versus concentrations. These lines were then used to calculate the unknown concentrations of II and III.

*Analysis of kinetic data*

The maximum velocity ($V_{max}$) and the Michaelis–Menten constant ($K_M$) values were evaluated by linear least-squares regression analysis using the Lineweaver–Burk plots. Values were expressed as means ± standard deviations.

## RESULTS AND DISCUSSION

*Chromatographic analysis*

Several gas chromatographic [9,17–25] and HPLC procedures [8, 26–29] have been described for the determination of mexiletine in biological fluids using a

variety of detection systems. However, the determination of II and III until now required two different chromatographic systems or previous derivatization before chromatography [12, 30], or was too insensitive [10]. In order to measure both metabolites simultaneously without derivatization, we have developed an HPLC procedure using fluorescence detection.

Fig. 2a illustrates a typical chromatogram of an aqueous standard solution containing 3 nmol/ml II and 2 nmol/ml III, Fig. 2b shows a chromatogram obtained from an extracted samples before incubation and Fig. 2c shows the results of extraction after incubation of human liver microsomes with mexiletine. According to the conditions previously described, the retention times were 3.8, 6.5, 11.7 and 15.5 min for metabolites III and II, the internal standard (IV) and mexiletine (I), respectively. Compared with blank incubations used as controls, all chromatograms were free from endogenous interferences. An additional peak (X) with a retention time of 4.9 min (unknown metabolite, Fig. 2c) was observed after incubation but did not interfere with the analysis.

The calibration graphs were obtained with standards incubated and extracted under the same conditions as described for the samples. Peak-height ratios of metabolites to internal standard were linearly related to their concentrations over the concentration ranges studied (0.6–6 nmol/ml for II and 0.3–3 nmol/ml for III). The linear regression equations were $y = -0.002 + 0.421x$ and

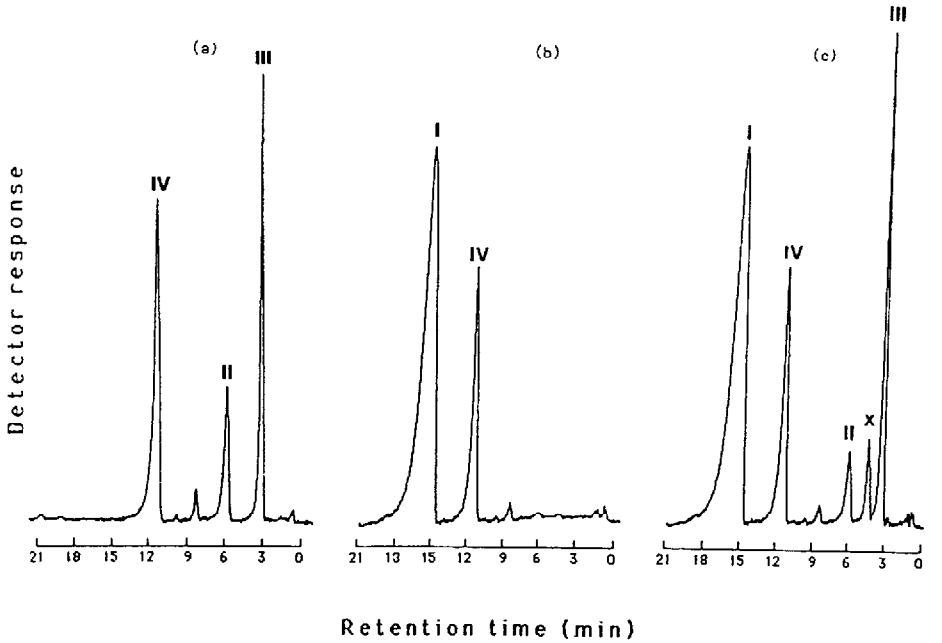


Fig. 2. Representative chromatograms of (a) an aqueous standard solution containing 3 nmol/ml hydroxymethylmexiletine (II) and 2 nmol/ml *p*-hydroxymetiletine (III), (b) an extracted sample before incubation and (c) after incubation of human liver microsomes with mexiletine (I) containing 2.2 nmol/ml hydroxymethylmexiletine (II) and 2.5 nmol/ml *p*-hydroxymexiletine (III). Peak IV = internal standard and X = unknown metabolite.

$y = -0.076 + 2.560x$ for II and III, respectively, with intercepts not significantly different from zero. The correlation coefficients for the regression lines were 0.9999 for II and 0.9977 for III.

The relative analytical recoveries of known concentrations of II and III (in the ranges 0.6–6 and 0.3–3 nmol/ml, respectively) were determined by comparing the peak-height ratios of extracted samples obtained without incubation with those of equivalent amounts of metabolites and internal standard dissolved in the mobile phase. The relative recoveries were $97 \pm 6\%$ for II and $87 \pm 5\%$ for III. The absolute recoveries were also determined for these concentration ranges using samples without incubation and treated using the procedure described above, except that the internal standard was omitted. All extraction sample residues were reconstituted in 100 $\mu$l of mobile phase solution containing 500 ng of internal standard. The absolute recoveries were $93 \pm 1\%$ for II and $78 \pm 6\%$ for III.

The detection limits were determined after extraction of the two metabolites and their internal standard added to the samples without incubation. For 25 $\mu$l injected on the column, the detection limits at a signal-to-noise ratio of 4:1 were 0.35 and 0.08 nmol/ml for II and III, respectively.

The intra-assay precision was established in a microsomal preparation containing the NADPH-generating system and spiked with II at concentrations of 1 and 6 nmol/ml and III at concentrations of 0.5 and 3 nmol/ml. For each concentration, the intra-assay precision of five consecutive runs was determined with coefficients of variation of 5.6 and 1.7% for II and 2.8 and 0.8% for III, respectively. The inter-assay precision was determined by analysing, on ten consecutive days, frozen aliquots from a similar microsomal preparation containing 1 nmol/ml II and 0.5 nmol/ml III. The mean concentration was found to be 1.04 nmol/ml for II and 0.5 nmol/ml for III. The coefficients of variation were 9.5% for II and 5.4% for III.

Under these conditions, the HPLC method allowed the production of the two metabolites of mexiletine in liver microsomal fractions to be evaluated simultaneously.

*Optimum incubation conditions*

The production of II and III by microsomal fractions, investigated with 330 $\mu M$ mexiletine concentrations and 1.4 mg/ml of microsomal proteins, increased up to 300 min with only a slight deviation from linearity (Fig. 3). The equations obtained from least-squares regression analysis of the data were $y = 0.061 + 0.008x$ and $y = 0.082 + 0.010x$ for II and III, respectively.

With a 240-min incubation time and a 330 $\mu M$ substrate, the reactions were linear with protein concentrations up to and including 2 mg/ml incubation (Fig. 4). The linear regression equations were $y = 0.010 + 0.0005x$ and $y = 0.004 + 0.006x$ for II and III, respectively.

Each time in these two studies and for the two metabolites, the correlation coefficients for the linear regression lines were better than 0.97 and significant ($p < 0.001$). The incubation conditions, therefore, would be well within the linear range for these two variables.

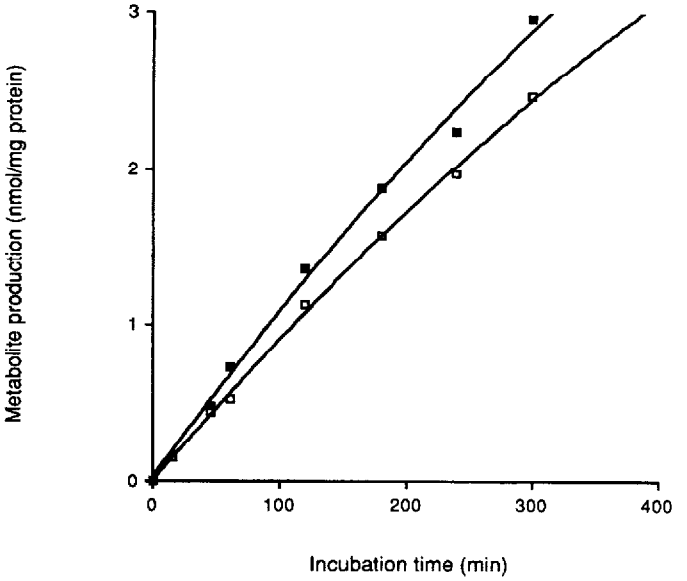


Fig. 3. Effect of time of incubation on the production of hydroxymethylmexiletine (□) and *p*-hydroxymexiletine (■) in microsomal fractions of human liver. Protein concentration, 1.4 mg/ml; mexiletine concentration, 330 $\mu M$.

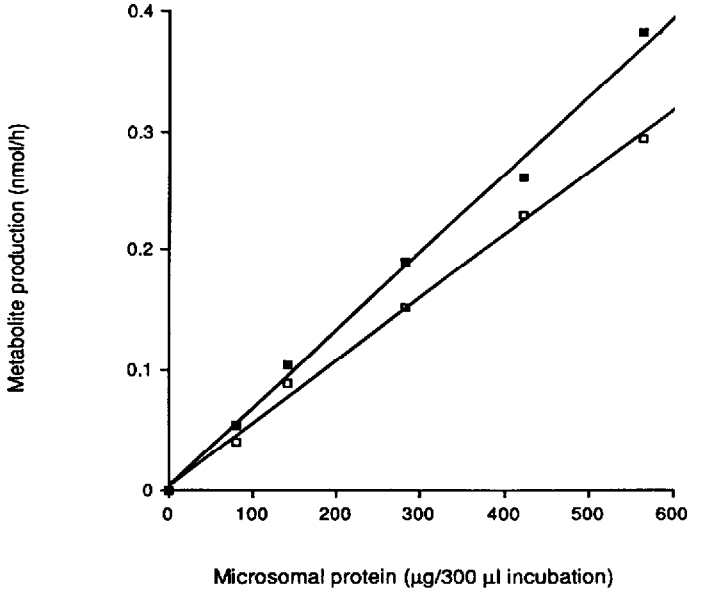


Fig. 4. Effect of protein concentration on the hydroxymethylmexiletine (□) and *p*-hydroxymexiletine (■) production in human liver microsomes. Time of incubation, 240 min; mexiletine concentration, 330 $\mu M$.

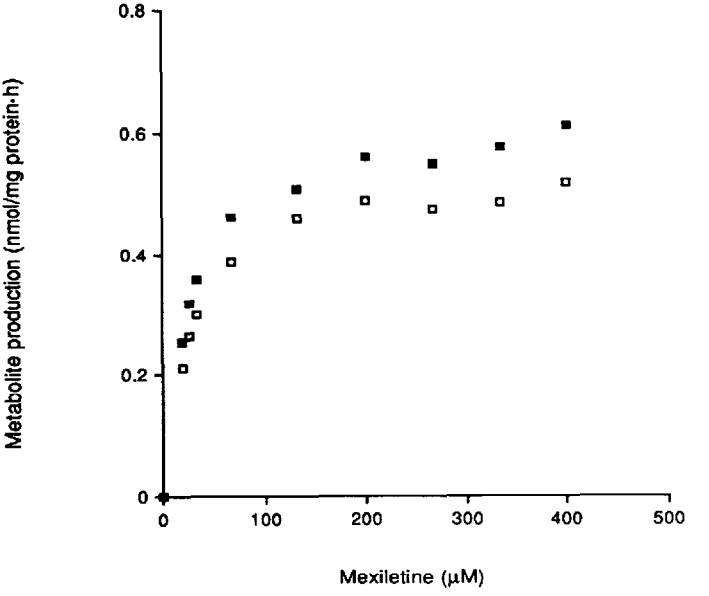


Fig. 5. Effect of varying mexiletine concentration (20–400 $\mu M$) on the hydroxymethylmexiletine (□) and *p*-hydroxymexiletine (■) production in human liver microsomes. Protein concentration, 1.4 mg/ml; time of incubation, 240 min.

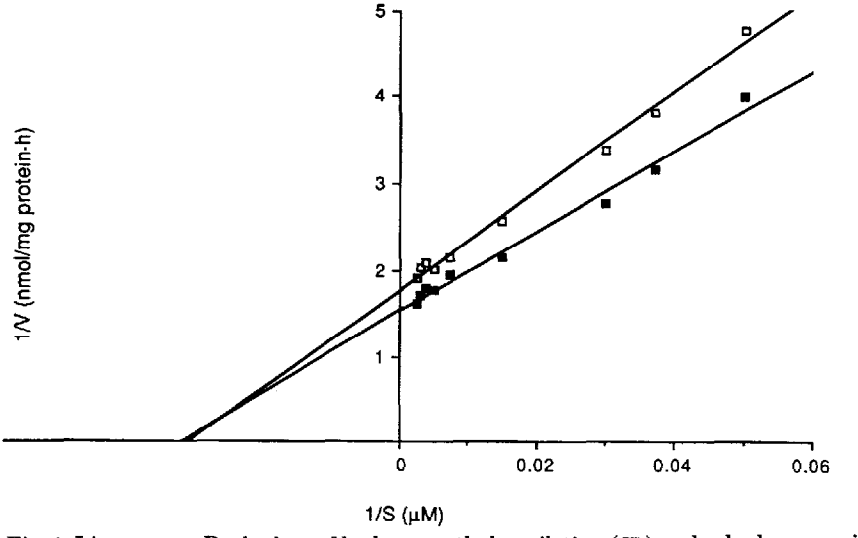


Fig. 6. Lineweaver–Burk plots of hydroxymethylmexiletine (□) and *p*-hydroxymexiletine (■) production in microsomal fraction from human liver. The linear regression equations are $y=1.770+56.416x$ and $y=1.553+45.514x$ for hydroxymethylmexiletine and *p*-hydroxymexiletine, respectively.

*Cofactor requirements*

The production of II and III was evaluated in duplicate in the absence of each reaction product. No metabolite peak was detected when the microsomal proteins, substrate or NADPH were omitted from the incubation.

*Reproducibility of the enzymatic reaction*

The reproducibility of the enzymatic reaction was investigated five times for the same microsomal preparation and for different microsomal preparations of the same liver at 330 $\mu M$ substrate and 1.4 mg/ml protein concentrations. The coefficients of variation were 3 and 14% for II and 4 and 13% for III, respectively.

*Kinetic study*

The effect of various concentrations of mexiletine ($S$) from 20 to 400 $\mu M$ on the production of II and III ($V$) by human liver microsomal fractions at 1.4 mg/ml of microsomal proteins was determined. Fig. 5 shows a typical result. The Lineweaver–Burk plots of the experimental data were linear (Fig. 6) with significant correlations between $1/V$ and $1/S$ ($r=0.9954$, $p<0.001$ and $r=0.9937$, $p<0.001$ for II and III, respectively). The enzymatic activities were assumed to follow monophasic Michaelis–Menten kinetics. The estimated Michaelis–Menten parameters for II and III were $K_M=31.8\pm1.8$ and $29.3\pm0.8$ $\mu M$, respectively, and the corresponding $V_{max}$ values were $0.56\pm0.6$ and $0.64\pm0.8$ nmol/mg protein/h.

## CONCLUSION

The HPLC assay described is simple and shows good reproducibility, sensitivity and selectivity. It allows the simultaneous kinetic characterization of the hydroxylation of mexiletine into its two major metabolites by human liver microsomes. This assay may be used in microsomal fractions from different human liver in order (i) to acquire a better knowledge of the kinetic behaviour of mexiletine and of its two major hydroxylated metabolites in patients and (ii) to investigate whether the large inter-individual variations of the apparent plasma kinetics of mexiletine are of metabolic origin and due to variations in its hydroxylation processes.

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## REFERENCES

1 C.Y.C. Chew, J. Collet and B.N. Singh, Drugs, 17 (1979) 161.
2 M. Gillis and R. Kates, Clin. Pharmacokin., 9 (1984) 375.
3 L.F. Prescott, A. Pottage and J.A. Clements, Postgrad. Med. J., 53 (Suppl. I) (1977) 50.
4 J.C. Peyrieux, J.P. Boissel, A. Leizorovicz and IMPACT Research Group, Fundam. Clin. Pharmacol., 1 (1984) 45.
5 N.P.S. Campbell, J.G. Kelly, A.A.J. Adgey and R.G. Shanks, Br. J. Clin. Pharmacol., 6 (1978) 103.
6 R.G. Talbot, R.A. Clark, J. Nimmo, J.M.M. Neilson, D.G. Julian and L.F. Precott, Lancet, ii (1973) 399.

7 R.G. Talbot, D.G. Julian and L.F. Precott, Am. Heart J., 91 (1976) 58.
8 W. Mastropaolo, D.R. Holmes, M.J. Osborn, R. Rooke and T.P. Moyer, Clin. Chem., 30 (1984) 319.
9 J. Vasiliades, J. Kelett and R.S. Cox, Am. J. Clin. Pathol., 81 (1984) 776.
10 O. Grech-Bélanger, M. Gilbert, J. Turgeon and P.P. LeBlanc, Clin. Pharmacol. Ther., 37 (1985) 638.
11 P.J. Pentikaïnen, I.H. Koivula and A.H. Hiltunen, Eur. J. Clin. Pharmacol., 23 (1982) 261.
12 A.H. Beckett and E.C. Chidomere, J. Pharm. Pharmacol., 29 (1977) 281.
13 A.H. Beckett and E.C. Chidomere, Postgrad. Med. J., 53 (Suppl. I) (1977) 60.
14 E.J. Begg, P.M. Chinwah, C. Webb, R.O. Day and D.N. Wade, Br. J. Clin. Pharmacol., 14 (1982) 219.
15 A.R. Boobis, M.J. Brodie, G.C. Kahn, D.R. Fletcher, J.H. Saunders and D.S. Davies, Br. J. Clin. Pharmacol., 9 (1980) 11.
16 O.H. Lowry, N.J. Rosebrough, A.L. Farr and R.J. Randa, J. Biol. Chem., 193 (1951) 265.
17 A. Pachecus, Y. Santoni, M. Fornaris, S. Magnan, C. Aubert, A. Ragon and J.P. Cano, Arzneim.-Forsch./Drug Res., 32 (1982) 688.
18 N. Szinai, R.J. Perchalski, R.H. Hammer, B.J. Wilder and R.R. Streiff, J. Pharm. Sci., 62 (1973) 1376.
19 R.J. Perchalski, B.J. Wilder and R.H. Hammer, J. Pharmc. Sci., 63 (1974) 1489.
20 S. Willox and B.N. Singh, J. Chromatogr., 128 (1976) 196.
21 D. Bradbrook, C. James and H.J. Rogers, Br. J. Clin. Pharmacol., 4 (1977) 380.
22 K.J. Smith and P.J.J. Meffin, J. Chromatogr., 181 (1980) 469.
23 A. Frydman, J.P. Lafarge, F. Vial, R. Ruliere and J.M. Alexandre, J. Chromatogr., 145 (1978) 401.
24 D.W. Holt, R.J. Flannagan, A.M. Hayler and M. Loizou, J. Chromatogr., 169 (1979) 295.
25 O. Grech-Bélanger, J. Chromatogr., 309 (1984) 165.
26 R. Kelly, D. Christmore, R. Smith, L. Doshier and S.L. Jacobs, Ther. Drug Monit., 3 (1981) 279.
27 H. Breithaupt and M. Wilfling, J. Chromatogr., 230 (1982) 97.
28 M.A. Andarelli, R. Farinotti and A. Dauphin, J. Pharm. Clin., 1 (1982) 113.
29 R.K. Bhamra, R.J. Flanagan and D.W. Holt, J. Chromatogr., 307 (1984) 439.
30 N.A. Farid and S.M. White, J. Chromatogr., 275 (1983) 458.