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SIMULTANEOUS DETERMINATION OF PERHEXILINE AND ITS MONOHYDROXY METABOLITES IN BIOLOGICAL FLUIDS BY GAS CHROMATOGRAPHY—ELECTRON-CAPTURE DETECTION

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SUMMARY

A rapid and sensitive method for the simultaneous determination of perhexiline and its *cis*-4-axial and *trans*-4-equatorial monohydroxy metabolites (M_1 and M_3, respectively) in human plasma, urine and bile is described. The assay utilises a single diethyl ether extraction, heptafluorobutyric acid anhydride derivatisation and separation and detection by gas chromatography—electron-capture detection. The limits of detection are 0.1 μg/ml for perhexiline and 0.025 μg/ml for the M_1 and M_3 metabolites. This method has been used in a five-day kinetic study of three healthy adult males who ingested a single 300-mg dose of perhexiline maleate. One of these volunteer subjects exhibited elevated plasma perhexiline and markedly reduced plasma and urinary M_1 concentrations together with profoundly prolonged plasma and urinary M_1 elimination times when compared with the other two subjects. These differences are thought to be of genetic origin. There were also obvious differences in urinary M_3 concentrations which were discussed.

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INTRODUCTION

Perhexiline maleate is an effective antianginal agent [1] whose therapeutic use is severely limited because of serious toxic side-effects including peripheral neuropathy [2] and hepatic damage with cirrhosis [3]. These side-effects, however, only appear to affect certain individuals who may exhibit genetic susceptibility [4]. Although a large number of assays for perhexiline have been reported [—10], in most instances they lack sensitivity and/or the ability to simultaneously measure the monohydroxyperhexilines or are relatively complex and laborious. This has consequently meant that there remains a lack of information regarding the metabolism and pharmacokinetics of the drug. A sensitive assay has been reported [11] together with a very limited pharmacokinetic investigation. This did appear to demonstrate inter-individual differences in metabolite production but continued for only 8—24 h, while previous studies had demonstrated prolonged plasma and urinary elimination half-lives [7, 10].

There was still, therefore, the need for appropriate pharmacokinetic assessment requiring frequent sampling over a prolonged period. The authors report a rapid, simple and sensitive assay for the simultaneous determination of perhexiline and its monohydroxy metabolites in biological fluids which has permitted fuller pharmacokinetic assessment of perhexiline maleate.

EXPERIMENTAL

Chemicals

Perhexiline maleate (reference compound MDL 3978G), [^{14}C]perhexiline maleate with specific activity 14.49 μCi/mg (MDL 3978G-49) and the 4-monohydroxyperhexiline metabolites M_1 (MDL 18452) and M_3 (MDL 10473) were donated by Merrell Dow Pharmaceuticals (Cincinnati, OH, U.S.A.). The internal standard, nortriptyline hydrochloride, was obtained from Eli-Lilly Pharmaceuticals (Basingstoke, U.K.). Tris—HCl was supplied by Sigma (Poole, U.K.). Diethyl ether, methanol, triethylamine, toluene and 880 ammonia, all of analytical grade, were obtained from B.D.H. (Poole, U.K.). Heptafluorobutyric acid anhydride (HFBAA) was obtained from Pierce (Chester, U.K.). All glassware was obtained from Baird and Tatlock (Poole, U.K.).

Gas chromatography (GC)

A Sigma-2 gas chromatograph (Perkin-Elmer, Bucks, U.K.), incorporating a ^{63}Ni electron-capture detector, was used with a silanised glass column (1.31 m × 2 mm I.D.) packed with 3% OV-7 on 100—120 mesh Gas Chrom Q (Phase Separations, Queensferry, U.K.). The column was conditioned at 150°C for 48 h, then at 215°C for 24 h with a carrier gas flow-rate of 40 ml/min. The optimum conditions were: column temperature 215°C, injection port temperature 250°C and detector temperature 300°C. The carrier gas, high-purity nitrogen, had a flow-rate of 40 ml/min through the column and was made up to 60 ml/min through the detector.

Analytical procedure

Extractions were carried out in 50-ml capacity glass stoppered test-tubes, pre-treated with dichlorodimethylsilane in toluene (2.5%, v/v) in order to minimise adsorption. To samples of plasma, urine and bile (2 ml) containing the internal standard nortriptyline (1 μg for plasma and 10 μg for urine and bile) were added 1 ml of 1.0 *M* Tris buffer (pH 10.6) and 20 ml of diethyl ether. The mixture was vortexed vigorously for 5 min and allowed to settle. The upper, organic layer was transferred to a clean test-tube and the ether allowed to evaporate to dryness at room temperature. The dry residue was re-constituted in 0.75 ml of fresh diethyl ether, transferred to a 3-ml stoppered vial and evaporated to dryness.

Derivatisation was accomplished by adding 12.5 μg of HFBAA and 0.5 ml of 0.05 *M* triethylamine in toluene, and heating at 75°C for 20 min. Upon cooling, excess HFBAA was hydrolysed with the addition of 1.5 ml of 5% aqueous ammonia. Following centrifugation (1000 *g* for 10 min), 0.25 ml of the upper toluene layer were transferred to a clean vial. An aliquot of 1—3 μl was injected into the gas chromatograph.

Human volunteer study

Ethical approval for this study was obtained from the Mersey Regional Health Authority Research Committee. Three normal, unrelated, male subjects gave informed consent for participation, there was no history of jaundice, hepatitis, alcohol excess or recent or current drug therapy in any subject. Personal details are given in Table I.

TABLE I

PERSONAL DETAILS OF THE NORMAL, MALE SUBJECTS INVESTIGATED

Subject	Age (years)	Height (m)	Weight (kg)
M.A.	25	1.79	80
R.C.	29	1.74	70
S.V.	24	1.80	74

Liver function was not formerly assessed but assumed to be normal in this healthy group. After an overnight fast, a single oral dose of perhexiline maleate, 300 mg, was swallowed with 300 ml water. A normal diet was allowed from 3 h after ingestion. Plasma samples were withdrawn from an indwelling multi-sampling venous catheter over a 120-h period and frequent urine samples were collected over timed intervals upto 120 h following ingestion and their volumes measured. Samples were stored at —20°C until analysis for perhexiline and monohydroxyperhexilines, M_1 and M_3.

RESULTS

The extraction yield of diethyl ether for perhexiline from water was 94.9 ± 2.1% calculated from the recovery of ^{14}C-radioactivity following extraction of

TABLE II

EXTRACTION EFFICIENCY OF DIETHYL ETHER FOR PERHEXILINE AND ITS MONOHYDROXY METABOLITES, M_1 AND M_3 FROM VARIOUS BODY FLUIDS COMPARED TO THAT FROM WATER

Concentration (μg/ml)	Extraction efficiency (mean ± S.D.) (%)		
	Perhexiline	M_1 metabolite	M_3 metabolite
Plasma			
0.5	88.3 ± 7.1	92.0 ± 5.2	85.0 ± 4.4
Urine			
0.5	96.2 ± 9.5	98.9 ± 5.8	95.5 ± 7.2
10	Not performed	89.3 ± 4.6	101.1 ± 4.3
Bile			
10	Not performed	96.2 ± 2.5	104.1 ± 4.2
50	101.7 ± 7.8	Not performed	Not performed

2-ml aliquots of water spiked with [^{14}C]perhexiline maleate, 10 000 dpm/ml, n = 10. The extraction efficiency of the solvent for perhexiline, M_1 and M_3 from plasma, urine and bile, compared with that from water at various concentrations, are seen in Table II. These results confirm previous reports of an efficient extraction [5]. Gas chromatograms of extracted and derivatised samples of drug-free plasma, urine and bile revealed no endogenous peaks likely to interfere with those of perhexiline or its metabolites. The retention time of perhexiline, M_1, M_3 and internal standard were 7.37, 8.24, 11.12 and 13.04 min, respectively. The compounds of interest to analyse were completely resolved from that of the internal standard (Fig. 1A). Urine samples collected from human volunteers, following ingestion of perhexiline maleate, produced five peaks in addition to those for perhexiline, M_1 and M_3 (Fig. 1B). Reference compounds for these peaks were not available but mass spectra obtained showed they were characteristic for perhexiline metabolites.

The limit of detection for perhexiline was 0.1 μg/ml, although the drug was detectable at concentrations as low as 0.05 μg/ml; for M_1 and M_3 the limit of detection was 0.025 μg/ml although both metabolites were easily detectable at concentrations as low as 0.01 μg/ml. A multiplicity of standard calibration curves were required in order to accommodate the large variations of concentrations of perhexiline, M_1 and M_3 in the various biological fluids. These standard curves were linear in the following analytical ranges: plasma perhexiline, M_1 and M_3 0.025—2.0 μg/ml (r = 0.992, 0.998 and 0.995, respectively), urinary perhexiline, M_1 and M_3 0.025—2.0 μg/ml (r = 0.992, 0.998 and 0.995, respectively) and 1—15 μg/ml (r = 0.993, 0.996 and 0.995, respectively) and biliary perhexiline 2.5—100 μg/ml (r = 0.991) and M_1 and M_3 1—15 μg/ml (r = 0.995 and 0.996, respectively).

Batches of samples of drug-free plasma, urine and bile were spiked with various concentrations of perhexiline, M_1 and M_3. Some of these batches of samples were stored at −20° C. Intra-assay coefficients were estimated by assaying unstored samples on the same day while inter-assay coefficients were

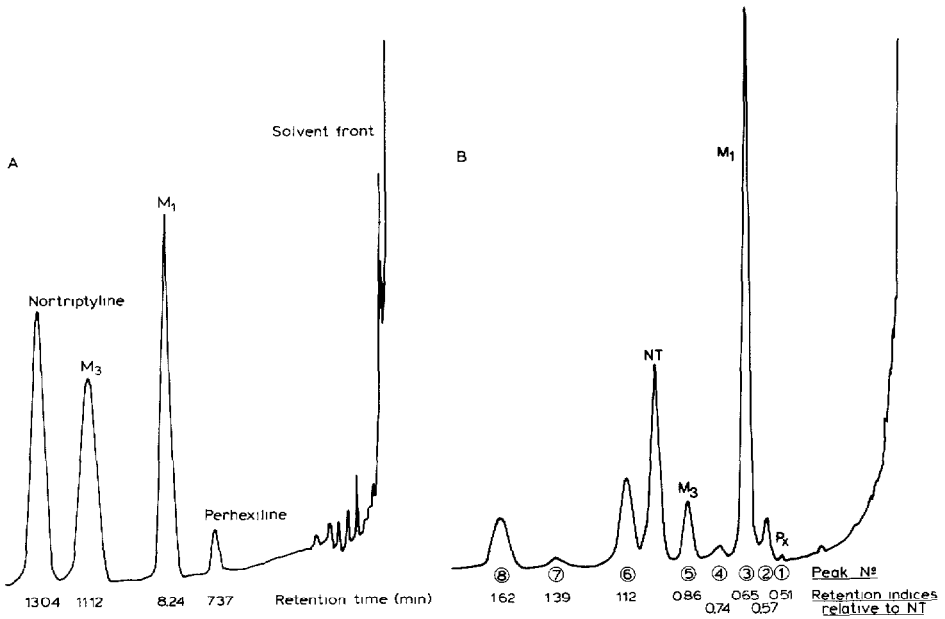

Fig. 1. (A) Gas chromatogram of extracted, derivatised plasma spiked with 5 μg/ml perhexiline, M_1 and M_3 metabolites together with nortriptyline (internal standard). (B) Gas chromatogram of extracted, derivatised urine collected between 12 and 24 h following single oral perhexiline dosage, 300 mg, in subject M.A.

TABLE III

INTRA-ASSAY COEFFICIENTS OF VARIATION FOR PERHEXILINE AND MONOHYDROXY METABOLITES EXTRACTED FROM VARIOUS BODY FLUIDS AT VARIOUS CONCENTRATIONS

Concentration (μg/ml)	Coefficient of variation (%)		
	Perhexiline	M_1 metabolite	M_3 metabolite
Plasma (n = 10)			
0.5	8.0	5.7	5.2
Urine (n = 10)			
0.1	15.5	12.2	12.1
0.5	10.0	5.9	4.4
2.5	9.4	8.0	8.4
10	5.1	5.2	2.1
Bile (n = 8)			
2.5	—	6.7	10.2
10	—	8.4	4.0
25	5.2	—	—
50	13.0	—	—

calculated from the results of stored samples which were assayed on different days over a period of two months. Intra- and inter-assay coefficients of variation are shown in Tables III and IV, respectively. There was no loss of concen-

TABLE IV

INTER-ASSAY COEFFICIENTS OF VARIATION FOR PERHEXILINE AND MONOHYDROXY METABOLITES EXTRACTED FROM VARIOUS BODY FLUIDS AT VARIOUS CONCENTRATIONS

Concentration (μg/ml)	Coefficient of variation (%)		
	Perhexiline	M_1 metabolite	M_2 metabolite
Plasma			
0.1	12.3 (n = 10)	1.8 (n = 5)	9.8 (n = 5)
0.5	3.2 (n = 10)	5.0 (n = 5)	6.4 (n = 5)
Urine			
0.1	12.3 (n = 10)	9.2 (n = 5)	10.5 (n = 5)
0.5	6.3 (n = 10)	9.4 (n = 5)	8.1 (n = 5)
2.5	6.9 (n = 16)	8.1 (n = 8)	7.5 (n = 8)
10	5.6 (n = 16)	6.0 (n = 8)	4.7 (n = 8)
Bile			
2.5	—	3.8 (n = 8)	3.5 (n = 8)
5	11.7 (n = 16)	—	—
10	—	2.8 (n = 8)	4.0 (n = 8)
20	7.2 (n = 16)	—	—

trations of perhexiline or monohydroxy metabolites during the storage period at −20°C.

Clinical studies

Plasma perhexiline concentrations during the initial 24 h in subjects M.A. and R.C. were close to or actually below the limit of detection and became undetectable within 48 h. Subject S.V., however, exhibited considerably elevated concentrations with a level at 120 h greater than that attained by subjects M.A. or R.C. at any time during the study. There were large variations in perhexiline concentrations during the first 36 h in subject S.V. with an elimination half-life, estimated after 36 h, of 65 h. It was impossible to estimate half-life data in subjects M.A. and R.C. Urinary perhexiline was at or below the limit of detection at all times in all subjects. The results of subject S.V. are seen in Fig. 2a.

Plasma M_1 concentrations rose rapidly in subjects M.A. and R.C., then declined gradually with half-lives estimated at 44 and 31 h, respectively. In subject S.V., plasma M_1 concentrations were markedly reduced and a half-life was inestimable since no significant decay occurred during the five-day study period (Fig. 2b).

Urinary M_1 concentrations rose rapidly in subjects M.A. and R.C. with considerable variability during the first 15 h after which there was a gradual decline with half-lives estimated at 38.5 and 37.5 h, respectively. The urinary M_1 concentrations in subject S.V. were markedly reduced with a half-life which was inestimable since no significant decay occurred during the study period (Fig. 2c).

Plasma M_3 was either on the limit of detection or undetectable in all subjects

throughout the study. Urinary M_3 levels rose rapidly in subjects M.A. and R.C. before declining gradually with half-lives estimated at 21.4 and 16 h, respectively. There was again considerable variability of results during the initial 15 h of the study. The urinary M_3 levels were less than the urinary M_1 levels in both of these subjects. In subject S.V. urinary M_3 excretion was initially less than in subjects M.A. and R.C. but after 48 h was actually greater than in those subjects. This is demonstrated in Fig. 2d which also indicates the extremely prolonged urinary M_3 elimination half-life which is inestimable during this study period. The urinary M_3 excretion in subject S.V. was clearly greater than the urinary M_1 excretion.

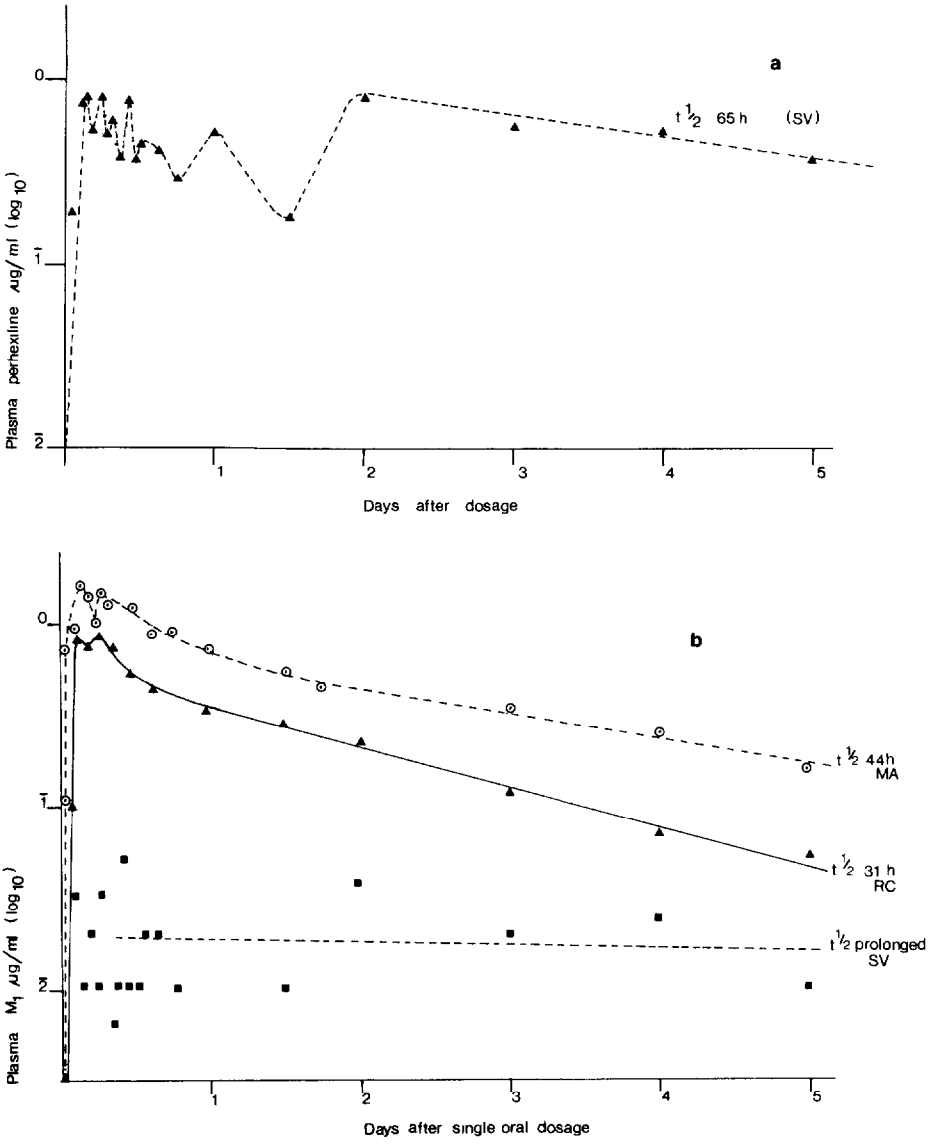

Fig. 2.

(Continued on p. 312)

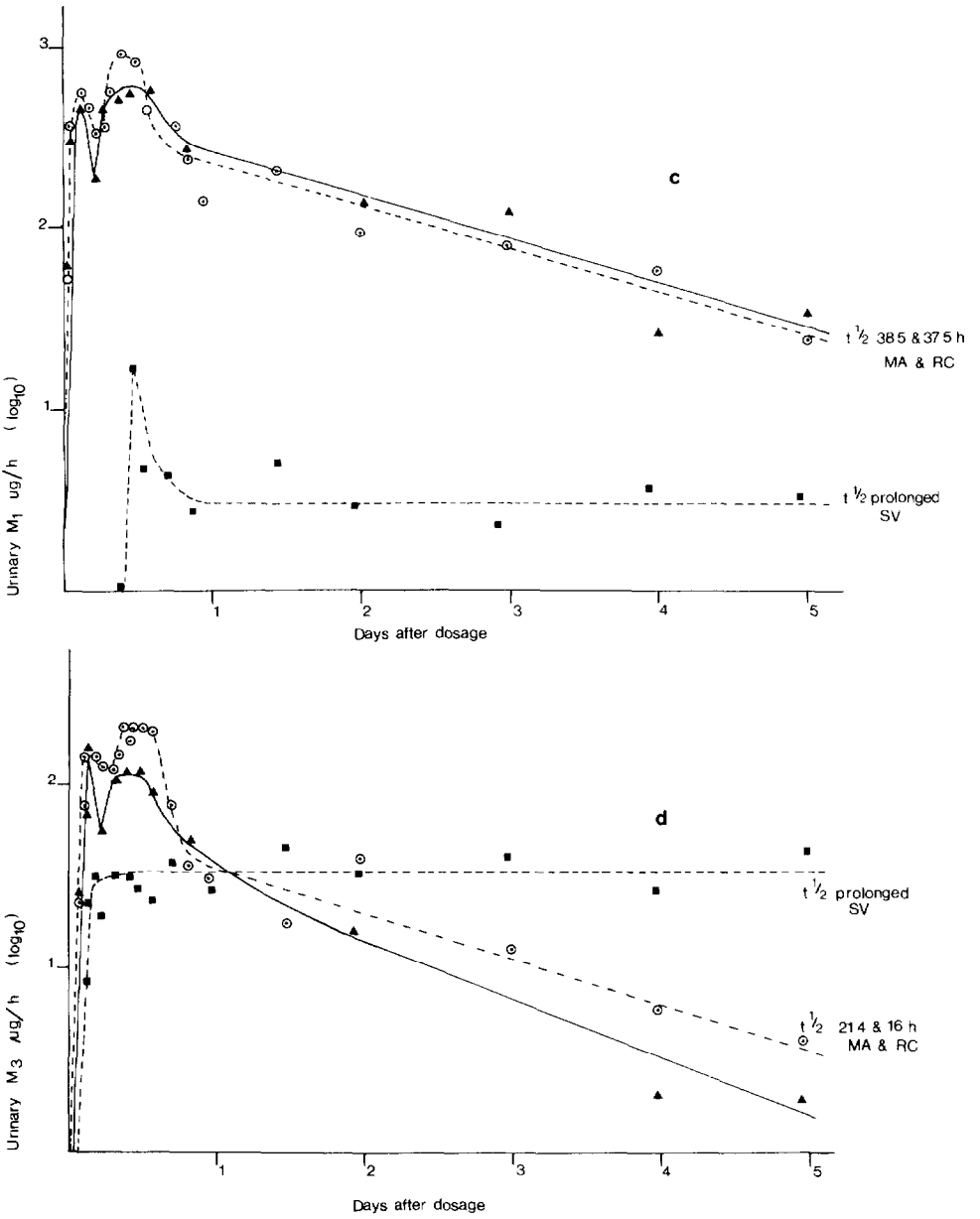

Fig. 2. Results from the five-day kinetic study following single oral perhexiline dosage. (a) Plasma perhexiline levels in subject S.V.; (b) plasma M_1 levels in all experimental subjects; (c) urinary M_1 excretion (μg/h) in all experimental subjects; (d) urinary M_3 excretion (μg/h) in all experimental subjects.

DISCUSSION

Diethyl ether has previously been shown to be an effective solvent for perhexiline and its metabolites [5], and this has been confirmed in this study. A most important step in the extraction procedure is that of alkalinisation, of the extraction mixture, in the present assay by Tris—HCl buffer at pH

10.6—10.75, since it has been previously demonstrated that the extraction efficiency declines markedly as extraction pH falls to below 7 [5]. Because of this basic extraction, no interference would be expected from common, non-prescribed, acidic compounds such as salicylic acid and paracetamol [5]. The use of diethyl ether for extraction, nortriptyline for internal standard, HFBAA for derivatization and gas chromatography—electron-capture detection are not novel but they have not previously been used in this combination, which has produced a reliable and repeatable assay. Although the absolute limits of detection are not quite as good as the best of previous methodology [11], this method represents a definite improvement on previous assays because of its simplicity and rapidity, its ability to simultaneously detect perhexiline and metabolites and its ability to determine perhexiline and metabolites in bile without the need for [^{14}C] perhexiline maleate. This assay has permitted a more extensive single-dose pharmacokinetic assessment of healthy human subjects.

Of the three subjects tested, two (M.A. and R.C.) had barely detectable plasma perhexiline levels and it was not possible to estimate a plasma half-life. The third subject (S.V.), however, had easily detectable plasma levels with a concentration at 96 h following single oral dosage of 0.51 μg/ml, almost as great as that found in fourteen non-neuropathic patients, 1.07 ± 0.19 μg/ml, who had ingested 200—300 mg of perhexiline daily for a mean of twenty months [7]. This subject, S.V., also had markedly reduced urinary M_1 excretion when compared with subjects M.A. and R.C. These differences were so great that the results required conversion to logarithmic form to accommodate all subjects on the same diagrams. Subject S.V. demonstrates a marked inability for perhexiline oxidation to its M_1 metabolite which is thought to be of genetic origin since his hepatic function is normal. This is not surprising since perhexiline oxidation polymorphism has already been confirmed on a population basis employing the "Perhexiline Oxidation Test" which compares individuals' 24 h plasma M_1 concentration and 12—24 h urinary M_1 excretion [12]. This study also confirmed that the genetic control for perhexiline oxidation polymorphism is identical to that for the debrisoquine/sparteine oxidation polymorphisms [12]. The interpretation of urinary M_3 results is a little more difficult. During the initial 24 h following ingestion, subject S.V., with markedly reduced M_1 production and excretion, had slightly reduced M_3 excretion but after 48 h had clearly greater M_3 excretion when compared with subjects M.A. and R.C. Subject S.V. has plasma M_1, urinary M_1 and urinary M_3 half-lives so prolonged that they are inestimable over a five-day study period. This has clear implications with regards to accumulation of the drug, in this subject and other individuals with impaired oxidation, on long-term therapy. Any repeat perhexiline pharmacokinetic studies would have to wait for prolonged periods, possibly many weeks, to allow complete excretion of this initial perhexiline dose, 300 mg, if misleading results are to be avoided.

It appears that in subject S.V. the oxidation pathway of perhexiline to both M_1 and M_3 is saturatable while a constant supply of oxidation substrate is supplied by elevated plasma perhexiline levels. As in previous studies [11], the urinary M_3 excretion in all subjects was greater than might have been expected for the observed plasma levels. The origin of variability of results during the early part of the study is not known, but variability of this nature has

previously been observed with compounds thought to be involved in enterohepatic circulation [13, 14].

REFERENCES

1 M. Afzal and E.M. Kafetzakis, Am. Heart J., 96 (1978) 350—354.
2 J. Nick, P. Dudognon, R. Escourolle, P. Bakouche, M.H. Nicholle, A Reignier, J.J. Hauw, S. Ermidou, S. Pollet, M. Baumann, E. Singlas and J. Levy, Rev. Neurol., 134 (1978) 103—114.
3 D. Pessayre, M. Bichara, G. Feldmann, C. Degott, F. Potet and J. Benhamou, Gastroenterology, 76 (1979) 170—177.
4 E. Singlas and P. Simon, Therapie, 36 (1981) 285—288.
5 J.D.H. Cooper and B.C. Turnell, Ann. Clin. Biochem., 17 (1980) 155—158.
6 J.F. Lang, R.A. Okerholm, J.D. Theile, B.J. Walker and G.J. Wright, Merrell Research Centre, Merrell National Laboratories, Cincinnati, OH, 1978.
7 E. Singlas, M.A. Goujet and P. Simon, Eur. J. Clin. Pharm., 14 (1978) 195—201.
8 N. Grgurinovich, J. Chromatogr., 274 (1983) 361—365.
9 J.D. Horowitz, P.M. Morris, O.H. Drummer, A.J. Goble and W.J. Louis, J. Pharm. Sci., 70 (1981) 320—322.
10 G.J. Wright, A V. Zeiger, G.A. Leeson and J.F. Lang, Postgrad. Med. J., 49 (Suppl. 3) (1973) 8—15.
11 A.G.B. Amoah, B.J. Gould and D.V. Parke, J. Chromatogr., 305 (1984) 401—409.
12 R.G. Cooper, D.A.P. Evans and E. Whibley, J. Med. Genet., 21 (1984) 27—33.
13 F.L.S. Tse, F. Ballard and J. Skinn, J. Pharmacokin. Biopharm., 10 (1982) 455—461.
14 H.G. Boxenbaum, G.S. Jodhka, A.C. Ferguson, S. Riegelman and T.R. MacGregor, J. Pharmacokinet. Biopharm., 2 (1974) 211—237.