Journal of Chromatography, 420 (1987) 287–296
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 3762

SIMULTANEOUS QUANTITATION OF PHENOBARBITONE AND *p*-HYDROXYPHENOBARBITONE AND THEIR PERDEUTEROETHYL ANALOGUES IN BIOLOGICAL FLUIDS BY COMBINED GAS CHROMATOGRAPHY–MASS SPECTROMETRY

Y. BENCHEKROUN, B. RIBON, M. DESAGE and J.L. BRAZIER*

Laboratoire d'Études Analytiques et Cinétiques du Medicament Faculté de Pharmacie, 8 Avenue Rockefeller, 69373 Lyon Cedex 08 (France)

(First received February 23rd, 1987; revised manuscript received April 22nd, 1987)

SUMMARY

In order to develop 5-pentadeuteroethyl-5-phenyl barbituric acid as an alternative tracer in pharmacokinetic and metabolic studies of phenobarbitone, and to search for possible isotope effects associated with such labelling, we propose a gas chromatographic–mass spectrometric assay for simultaneous measurement of phenobarbitone, *p*-hydroxyphenobarbitone and their perdeuteroethyl analogues, using [1,3-$^{15}N_2$,2-^{13}C] phenobarbitone as internal standard. These compounds were extracted from plasma (50 μl) or urine (500 μl) and pentylated according to Greeley's method. Linear calibration curves were obtained in the concentration range from 0.5 to 3 μg/ml. The interday precision, mean accuracy and detection limit were 0.77–5.28%, 99.99–100.80% and 0.03–0.05 μg/ml, respectively. Results for plasma and urine concentrations, and pharmacokinetic parameters in humans, are presented to illustrate this method.

INTRODUCTION

Numerous methods are available for the quantitation of phenobarbitone (PB) alone or in the presence of other antiepileptic drugs in plasma. In contrast, relatively few methods [1–7] have been reported for the simultaneous analysis of PB and its known major metabolite *p*-hydroxyphenobarbitone (pHPB). However, these methods are tedious and/or suffer from poor detection limits (ca. 1 μg/ml).

In recent years increased analytical performance has been made possible thanks to use of gas chromatography–mass spectrometry (GC–MS) [8–11] and stable isotopically labelled internal standards such as [1,3-$^{15}N_2$,2-^{13}C]PB and [1,3-$^{15}N_2$,2-^{13}C]pHPB [9], or 5-ethyl-5-(2,3,4,5-tetradeuterophenyl)-[2-^{13}C]-barbituric acid and 5-ethyl-5-(4-hydroxy-3,5-dideuterophenyl)-[1,3-$^{15}N_2$,2-^{13}C]-barbituric acid [10].

0378-4347/87/$03.50

As well as permitting excellent analytical performance [12,13] these compounds may be used as tracers in a large array of biomedical studies [14], especially for determining the steady-state pharmacokinetics of drugs in patients on monotherapy and studies on the interactive pharmacokinetics of these drugs under combined drug therapy [15–17], which is frequently observed for antiepileptics.

To our knowledge only [1,3-$^{15}N_2$,2-^{13}C]PB, a relatively expensive molecule, has been proposed as a stable isotopically labelled tracer of PB [10,18].

In order to develop 5-pentadeuteroethyl-5-phenyl barbituric acid (PBd5), a much less expensive analogue, as an alternative tracer in PB pharmacokinetic and metabolic studies, and to search for possible isotope effects associated with such a labelling, we propose a technique allowing simultaneous measurement of two sets of compounds, PB and PBd5 on one hand and pHPB and pHPBd5 on the other, from single plasma or urine samples, with [1,3-$^{15}N_2$,2-^{13}C]PB serving as analytical internal standard.

EXPERIMENTAL

Materials

PB and pHPB were purchased from Sigma (St. Louis, MO, U.S.A.) and Aldrich-Chimie (Strasbourg, France), respectively. PBd5 (isotopic enrichment > 99%) and [1,3-$^{15}N_2$,2-^{13}C]PB were synthesized by Commissariat à l'Énergie Atomique (CEA, Saclay, France). Tetramethylammonium hydroxide, N,N-dimethylacetamide, 1-iodopentane, chloroform, 2-propanol, diethyl ether, toluene and ethyl acetate (analytical-grade quality) were purchased from Merck (Darmstadt, F.R.G.) and used without further purification. Acetate buffer (0.1 *M*, pH 5.2) was prepared from 0.1 *M* sodium acetate and 0.1 *M* acetic acid. *Helix pomatia* enzymic extract (minimal activities: 100 000 I.U./ml for glucuronidase, 1 000 000 I.U./ml for sulphatase) was purchased from Pharmindustrie (Genevilliers, France).

Stock 100 μg/ml ethanolic solutions of PB, PBd5, internal standard and pHPB were prepared. All stock solutions were stored at 4 °C and used within 4 weeks. Fresh working solutions of 10 μg/ml were prepared by ten-fold dilution of stock solutions in water.

Extraction

Plasma. A 200-μl volume of acetate buffer (pH 5.2) and 50 μl of internal standard solution (10 μg/ml) were added to 50 μl of plasma in 15-ml conical centrifuge tubes. After mixing and addition of 2 ml of extraction solvent (chloroform–2-propanol, 95:5), the samples were extracted using a vortex mixer (1 min). Following centrifugation the organic phase was recovered and evaporated to dryness under a stream of nitrogen at 40 °C.

Urine. To 500-μl samples in 50-ml screw-cap centrifuge tubes were added successively 100 μl of internal standard solution (10 μg/ml) and 500 μl of acetate buffer (pH 5.2). After mixing, two drops of *H. pomatia* enzymic extract were added; the resulting mixture was vortexed and incubated at 37 °C for 20 h. Com-

pounds were then extracted at ambient temperature (vortex mixer, 90 s) using 5 ml of diethyl ether. After centrifugation the organic phase was recovered and evaporated to dryness under a stream of nitrogen at 40°C.

Derivatization of N–H and O–H groups

Derivatization was performed according to the procedure described by Greeley [19]. First, 100 μl of N,N-dimethylacetamide and 50 μl of tetramethylammonium hydroxide solution (0.1 *M*) were added to the dry residue. The mixture was shaken vigorously for 10 s, and 50 μl of iodopentane were then added. The solution was shaken again then allowed to stand at room temperature for 10 min. Following centrifugation, the organic phase was recovered and evaporated to dryness. The dry residue was dissolved in 100 μl of toluene–ethyl acetate mixture (5:2); the resulting sample was suitable for GC–MS analysis.

Chromatographic separation

The gas chromatograph was a Hewlett-Packard Model 5790 equipped with an automatic injector HP 7672A. The capillary column used was an HP OV 1 cross-linked dimethyl silicone fused-silica column (12 m×0.22 mm I.D.). Samples (1 μl) were automatically injected into the chromatograph in the splitless mode. The splitless valve time was 0.75 min. Injector temperature was set at 250°C, and the oven temperature was programmed at 20°C/min from 150°C (0.8 min) to 260°C (5 min). The transfer line temperature was 260°C. Helium was used as carrier gas at a flow-rate of 2 ml/min.

Detection and measurements

A Hewlett-Packard 5970A mass-selective detector was used for detection. Ions monitored were: *m/z* 303 (PB); 306 ([1,3-$^{15}N_2$,2-^{13}C]PB); 308 (PBd5); 458 (pHPB) and 463 (pHPBd5). Dwell time was 50 ms in all cases. The detector was operating between 7.5 and 13 min. Areas were integrated according to the horizontal mode (5% slope sensitivity).

Standardization

The quality of the method was investigated for the analysis of PB and PBd5 in plasma, and for PB, PBd5 and pHPB (following enzymic hydrolysis using a procedure derived from Whyte and Dekaban [3] in urine samples. For this purpose various samples spiked with PB, PBd5 and pHPB at concentrations of 0.5, 1, 2, and 3 μg/ml were prepared and treated as described above. pHPBd5 was not available as a reference compound, but its chemical behaviour was assumed to be identical with that of pHPB.

RESULTS

Mass spectra and fragmentograms

The mass spectra of N,N-dipentyl derivatives of PB, [1,3-$^{15}N_2$,2-^{13}C]PB, and PBd5, and N,N,O-tripentyl derivatives of pHPB and pHPBd5 are shown in Fig. 1 (the last of these was obtained from a urine sample from a PBd5-treated patient); *m/z* values for the molecular ions and base peaks are, respectively: 372

and 146 (PB); 375 and 146 (internal standard); 377 and 308 (PBd5); 458 and 162 (pHPB); 463 and 167 (pHPBd5). Monitored ions were chosen according to three major criteria: high relative intensity and mass, and optimal simplicity of isotopic clusters.

Fig. 2 shows a fragmentogram of a urine sample extract from a patient treated with a mixture of PB and PBd5. Under such conditions, the retention times were 8.12 min for PBd5; 8.15 min for PB and [1,3-$^{15}N_2$,2-^{13}C]PB; 12.28 min for pHPBd5; and 12.31 min for pHPB.

Linearity

Spiked samples were measured five times each under the conditions described above. A linear regression analysis of the values (peak-area ratio vs. concentration) indicated an almost linear fit of the data:

In plasma,	PB	: slope = 1.081; intercept = 0.048; r = 1.0000
	PBd5	: slope = 1.328; intercept = 0.081; r = 1.0000
In urine,	PB	: slope = 1.131; intercept = 0.018; r = 0.9999
	PBd5	: slope = 1.772; intercept = 0.049; r = 0.9998
	pHPB	: slope = 0.931; intercept = 0.006; r = 0.9997

Accuracy and precision

The accuracy and reproducibility of the method were evaluated for each concentration value (0.5, 1, 2, 3 μg/ml) by measuring urine concentrations once a week and plasma concentrations twice a week over 6 weeks. The results are summarized in Table I.

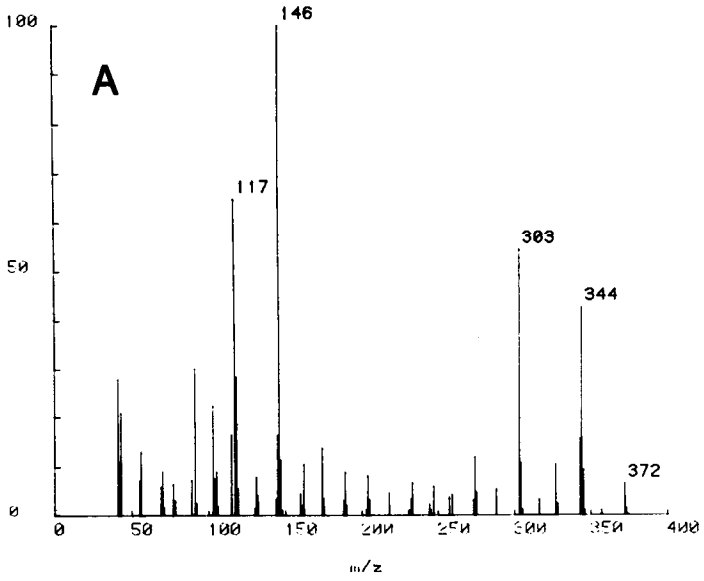
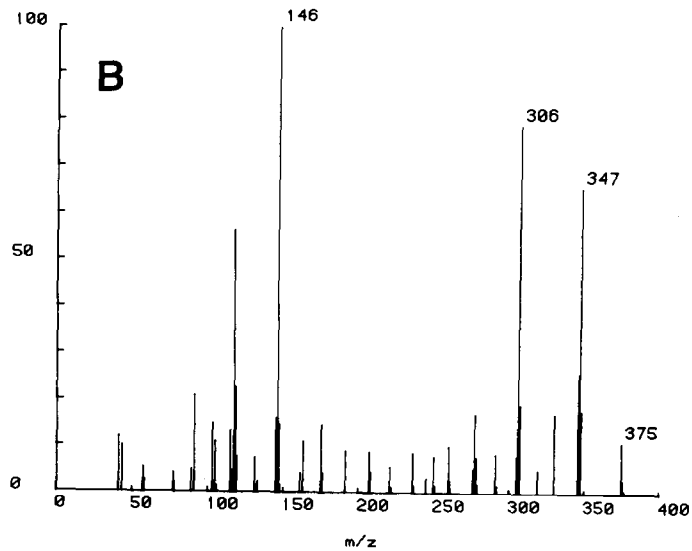


Fig. 1.



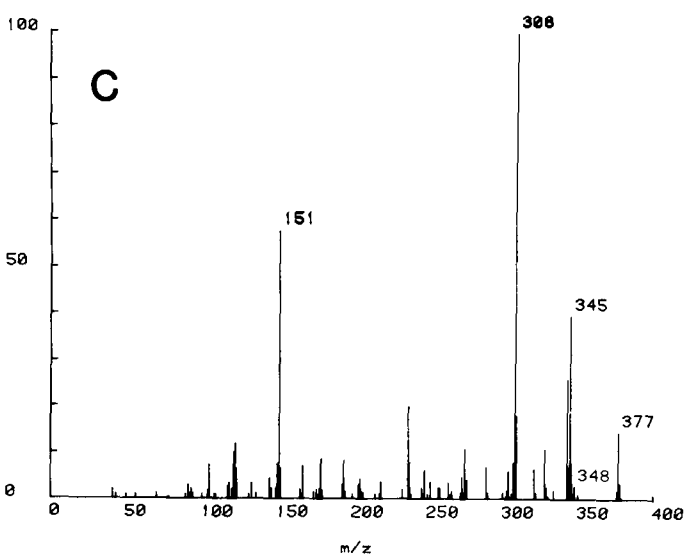


Fig. 1.

(Continued on p. 292)

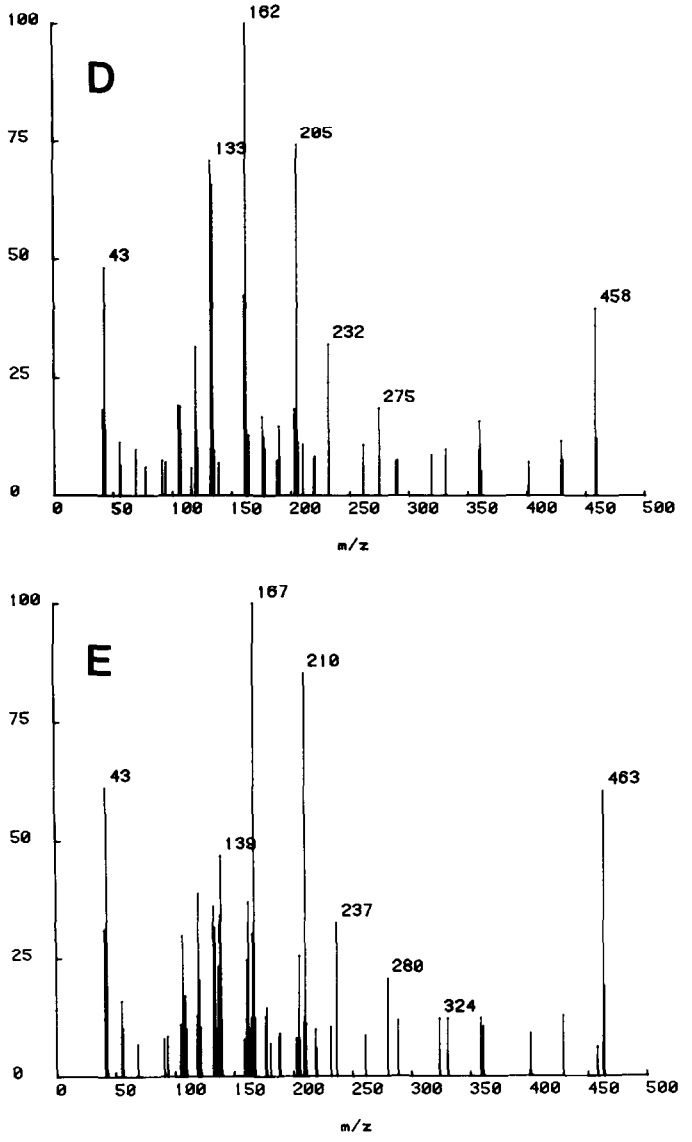


Fig. 1. Mass spectra of N,N-dipentyl derivatives of (A) PB; (B) [1,3-$^{15}N_2$,2-^{13}C]PB; and (C) PBd5, and of N,N,O-tripentyl derivatives of (D) pHPB and (E) pHPB d5.

Detection limit

For each compound the detection limit was taken as the lowest concentration yielding an isotopic contribution corrected area of 50 electron-multiplier-counts, i.e. ca. 10-fold the analytical noise obtained on injecting plasma or urinary blanks. Isotopic contribution, which stems from both the natural isotopic abundance and labelled compounds isotopic impurities, was assessed by repeatedly measuring the three ions of interest from each of the PB isotopomers, namely *m/z* 303, 306 and 308. Results of these measurements, expressed as percentages of the area of the monitored ions for each of the assayed molecules, are presented in Table II.

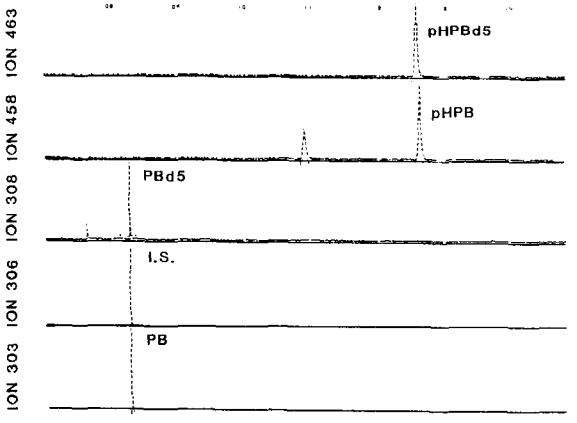


Fig. 2. Typical fragmentograms of ions 303 (PB), 306 (I.S.), 308 (PBd5), 458 (pHPB) and 463 (pHPBd5), from an urine sample extract.

TABLE I

ACCURACY AND PRECISION

Matrix	Compound	Spiked concn. (μg/ml)	Measured concn. (mean ± S.D.) (μg/ml)	Precision (R.S.D.) (%)	Accuracy (%)	
					A★	Mean ± S.D.
Plasma ($n=12$)	PB	0.5	0.497 (0.015)	2.93	99.40	
		1.0	1.007 (0.025)	2.45	100.70	100.03
		2.0	1.999 (0.023)	1.13	99.95	(0.53)
		3.0	3.002 (0.029)	0.98	100.07	
	PBd5	0.5	0.503 (0.037)	2.28	100.60	
		1.0	0.997 (0.015)	1.54	99.70	100.10
		2.0	2.001 (0.017)	0.87	100.05	(0.37)
		3.0	3.001 (0.023)	0.77	100.03	
Urine ($n=6$)	PB	0.5	0.498 (0.017)	3.37	99.60	
		1.0	1.006 (0.020)	1.95	100.60	100.07
		2.0	2.005 (0.027)	1.37	100.25	(0.44)
		3.0	2.995 (0.046)	1.54	99.83	
	PBd5	0.5	0.499 (0.011)	2.20	99.80	
		1.0	1.006 (0.022)	1.57	100.60	99.99
		2.0	1.983 (0.039)	1.98	99.15	(0.65)
		3.0	3.012 (0.047)	1.56	100.40	
	pHPB	0.5	0.515 (0.026)	4.98	103.00	
		1.0	0.992 (0.05)	5.03	99.20	100.80
		2.0	2.023 (0.107)	5.28	101.50	(1.79)
		3.0	2.985 (0.060)	1.94	99.50	

★$A = 100 - 100 \times \frac{\Delta x}{x}$.

TABLE II

MEAN CALCULATED ISOTOPIC CONTRIBUTION VALUES

Average of five measurements.

Molecule (m/z)	PB %303	I.S. %306	PBd5 %308
303	100%	0.61%	1.69%
306	0%	100%	0.48%
308	0%	5.25%	100%

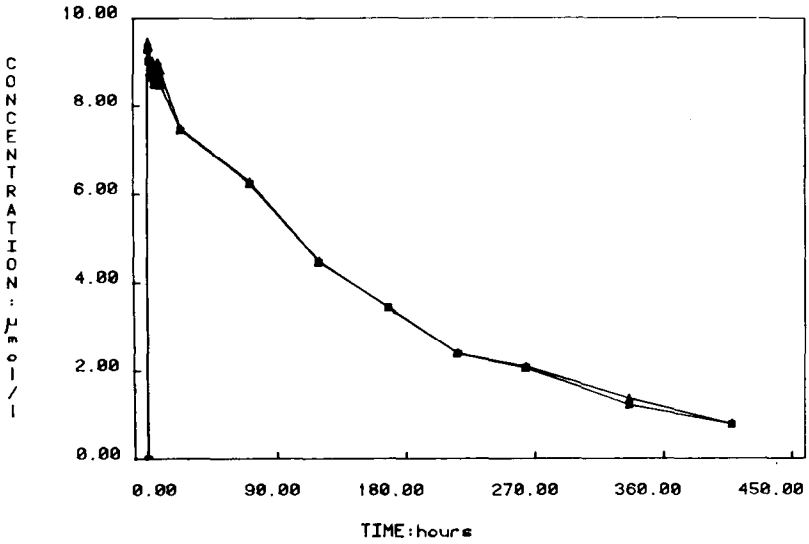


Fig. 3. Time-course of plasma concentrations of (▲) PB and (■) PBd5 after oral administration to a healthy male volunteer (age 37 years, weight 88 kg) of a single dose of a mixture of PB and PBd5 (1.24 mg/kg of each compound).

TABLE III

HUMAN PHARMACOKINETIC PARAMETERS

k_e = Elimination constant; $t_{\frac{1}{2}}$ = half-life; AUC = area under the curve; Clt = total clearance; V_d = volume of distribution.

	Dose		k_e (1/h)	$t_{\frac{1}{2}}$ (h)	AUC (mg/l·h)	Clt (l/h)	V_d (l/kg)
	(mg)	(mg/kg)					
PB	109.44	1.24	0.0059	117.9	361.1	0.303	0.589
PBd5	109.24	1.24	0.0059	117.3	363.4	0.301	0.579

TABLE IV

CUMULATIVE URINARY ELIMINATION AS A PERCENTAGE OF DOSE

		Time intervals (h)				
		0-12	12-24	24-36	36-60	60-84
Non-hydrolysed samples	PB	1.62	2.17	3.47	5.31	8.02
	PBd5	1.50	2.03	3.28	5.07	7.71
	pHPB	0.15	0.34	0.56	0.96	1.52
	pHPBd5	0.16	0.36	0.60	1.03	1.63
Hydrolysed samples	pHPB	0.26	0.60	0.93	1.63	2.62
	pHPBd5	0.28	0.65	1.02	1.80	2.89

In cases where these three compounds are simultaneously present in a given sample, and neglecting the 0.48% isotopic contribution of PBd5 in ion $m/z = 306$ corresponding to the internal standard, measured areas may be corrected according to the following formulae:

$$(308 \text{ ion area})_{\text{cor.}} = (308 \text{ ion area}) - (0.0525 \times 306 \text{ ion area})$$

$$(303 \text{ ion area})_{\text{cor.}} = (303 \text{ ion area}) - (0.0061 \times 306 \text{ ion area}) - (0.0169 \times (308 \text{ ion area})_{\text{cor.}})$$

As for pHPB and pHPBd5, the mass difference (5 daltons) between the two monitored ions is large enough for the isotopic contribution to be neglected. Under such conditions, calculation of the detection limits yields following results:

Plasma PB and PBd5 : 2 ng (0.04 μg/ml)
Urine, PB; PBd5 : 25 ng (0.05 μg/ml)
pHPB : 15 ng (0.03 μg/ml)

DISCUSSION

The proposed method offers several advantages in terms of sensitivity (detection limit < 0.05 μg/ml), specificity and ability to assay PB and its hydroxylated metabolite simultaneously in biological fluids. Moreover, the sample preparation procedure described is relatively simple and rapid compared with the approach of Patel et al. [9], and a single isotopically labelled internal standard makes the technique less expensive than those previously proposed by Patel et al. [9] and Van Langenhove et al. [10].

To conclude, this method makes it possible to use PBd5 in the course of pharmacokinetic and metabolic studies, thus reducing the cost of such studies with the single condition this labelled isotopomer is free from metabolic isotope effects, a point we also investigated in humans following oral administration of a mixture of PB and PBd5 (1.24 mg/kg of each compound): pharmacokinetic parameters, and plasma and urine concentrations of the relevant molecules are presented in Fig. 3 and Tables III and IV.

ACKNOWLEDGEMENT

The authors are grateful to Dr. J.B. Falconnet, for helpful suggestions concerning the manuscript.

REFERENCES

1 T.C. Butler, J. Pharmacol. Exp. Ther., 116 (1956) 326.
2 N. Kallberg, S. Agurell, O. Ericsson, E. Bucht, B. Jalling and L.O. Boreus, Eur. J. Clin. Pharmacol., 9 (1975) 161.
3 M.P. Whyte and A.S. Dekaban, Drug Metab. Dispos., 5 (1977) 63.
4 S.S. Levin, M.F. Schwartz, D.Y. Cooper and J.C. Touchstone, J. Chromatogr., 154 (1978) 349.
5 R.W. Dykeman and D.J. Ecobichon, J. Chromatogr., 162 (1979) 104.
6 F. Sarhan, J.M. Ziegler, A. Nicolas and G. Siest, J. Chromatogr., 183 (1980) 505.
7 R. Riva, F. Albani and A. Barruzi, J. Chromatogr., 221 (1980) 75.
8 H. Nau, D. Jesdinsky and W. Wittfoht, J. Chromatogr., 182 (1980) 71.
9 I.H. Patel, R.H. Levy, J.M. Neal and W.F. Trager, J. Pharm. Sci., 69 (1980) 1218.
10 A. van Langenhove, J.E. Biller, K. Biemann and T.R. Browne, Biomed. Mass Spectrom., 9 (1982) 201.
11 I.M. Kapetanovic and H.J. Kupferberg, J. Pharm. Sci., 70 (1981) 1218.
12 M. Desage, J.L. Brazier, F. Comet and R. Guilluy, Analusis, 11 (1983) 119.
13 N.J. Hasking, Biomed. Mass Spectrom., 9 (1982) 269.
14 T.A. Baillie, Pharm. Rev., 33 (1981) 81.
15 L. Bertilsson and T. Tomson, J. Clin. Pharmacol., 26 (1986) 459.
16 T.R. Browne, D.J. Greenblatt and J.E. Evans, J. Clin. Pharmacol., 26 (1986) 463.
17 M. Eichelbaum, J. Clin. Pharmacol., 26 (1986) 469.
18 I.M. Kapetanovic and H.J. Kupferberg, Biomed. Mass Spectrom., 7 (1980) 47.
19 R.H. Greeley, J. Chromatogr., 88 (1974) 229.