Journal of Chromatography, 616 (1993) 253–259
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam

CHROMBIO. 6870

High-performance liquid chromatographic method for resolving the enantiomers of mexiletine and two major metabolites isolated from microbial fermentation media

D. G. Freitag, R. T. Foster, R. T. Coutts and F. M. Pasutto*

Faculty of Pharmacy and Pharmaceutical Sciences, University of Alberta, Edmonton, Alberta T6G 2N8 (Canada)

(First received January 27th, 1993; revised manuscript received April 2nd, 1993)

ABSTRACT

(±)-Mexiletine is a class Ib antiarrhythmic drug useful in the treatment of premature ventricular contractions. It is predominantly metabolized by the liver with less than 15% being excreted in urine as unchanged drug. *p*-Hydroxymexiletine (PHM) and hydroxymethylmexiletine (HMM) are the two major mammalian metabolites. The purpose of our study was to develop a stereospecific high-performance liquid chromatographic (HPLC) method to determine whether the fungus, *Cunninghamella echinulata* (UAMH 4145), was able to biosynthesize these same two metabolites from the substrate (±)-mexiletine. Furthermore, it was desirable to ascertain whether metabolism of mexiletine was stereoselective. The method requires pre-column derivatization of the drug and metabolites with *S*-(+)-1-(1-naphthyl)ethyl isocyanate (NEIC) followed by normal-phase HPLC. Mexiletine, PHM, HMM and (±)-1-(4-hydroxyphenoxy)-3-isopropylaminopropan-2-ol (internal standard) were extracted from microbial broth using two volumes of diethyl ether after basifying with sodium carbonate. The combined ether extracts were evaporated to dryness, using a gentle stream of nitrogen, and reconstituted in 0.3 ml of chloroform to which was added 0.075 ml of NEIC (0.1%, v/v, in chloroform). This solution was immediately evaporated to dryness under a nitrogen stream. The residue was reconstituted with 0.220 ml of chloroform and 0.030 ml of *n*-butylamine (0.33%, v/v, in chloroform) and injected into the HPLC system.

INTRODUCTION

(±)-Mexiletine (Fig. 1a) is a class 1b antiarrhythmic drug used in the treatment of ventricular arrhythmias. It is similar in structure to lidocaine (half-life is 1 h), yet is effective orally due to a longer elimination half-life ranging from 6 to 12 h [1]. Therapeutic serum concentrations range from 0.5 to 2.0 μg/ml and levels greater than 2.0 μg/ml can cause neurological side-effects [2,3]. Unlike lidocaine, (±)-mexiletine possesses a chiral center with the individual enantiomers displaying differences in pharmacokinetics [4,5], receptor binding [6] and electrophysiology [7]. (±)-Mexiletine is eliminated primarily through hepatic metabolism with up to 15% of a single peroral dose, administered as a racemate, being recovered unchanged in urine [8]. The major mammalian metabolites are hydroxymethylmexiletine (HMM, Fig. 1b) and *p*-hydroxymexiletine (PHM, Fig. 1c) [9].

Numerous methods have been developed for the analysis of racemic mexiletine in the absence of metabolites. These include gas chromatographic (GC) [10–13] and reversed-phase high-performance liquid chromatographic (HPLC) methods for derivatized [14–20] or underivatized drug [21–24]. Racemic mexiletine and metabolites have been analyzed using GC [9,25–27] and reversed-phase HPLC with [28,29] or without

* Corresponding author.

0378-4347/93/$06.00

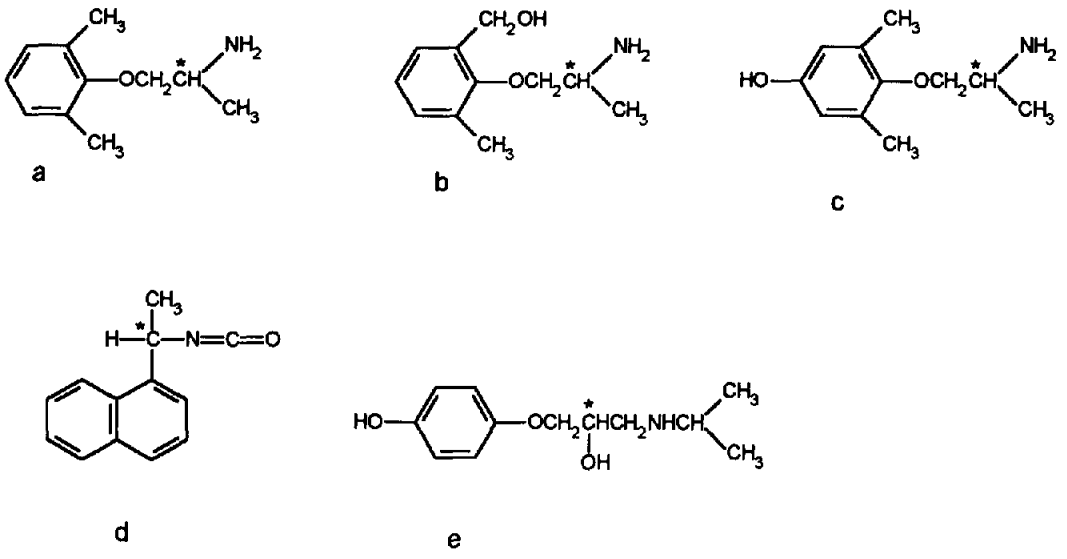

Fig. 1. Structures of (a) mexiletine, (b) HMM, (c) PHM, (d) NEIC and (e) racemic prenalterol. The asterisk denotes chiral center.

[30–34] derivatization. Stereospecific analyses for mexiletine alone include resolution of the diastereomers by GC [4], reversed-phase HPLC [4,7,35–37], or direct separation on a Pirkle-type stationary phase [38,39].

The purpose of our study was to design an analytical technique that would allow us to measure the individual enantiomer concentrations of mexiletine, HMM, and PHM simultaneously. A normal-phase HPLC method, utilizing pre-column derivatization with the enantiopure reagent *S*-(+)-1-(1-naphthyl)ethyl isocyanate (NEIC, Fig. 1d), was developed. This assay was used to determine whether the fungus, *Cunninghamella echinulata* (UAMH 4145), was able to biosynthesize the major mammalian metabolites, HMM and PHM, from the substrate (±)-mexiletine.

EXPERIMENTAL

Chemicals

(±)-Mexiletine hydrochloride, *R*-(−)-mexiletine hydrochloride, *S*-(+)-mexiletine hydrochloride, (±)-HMM hydrochloride and (±)-PHM oxalate were kindly donated (Boehringer Ingelheim, Burlington, Canada). The internal standard (±)-1-(4-hydroxyphenoxy)-3-isopropylaminopropan-2-ol (racemic prenalterol, Fig. 1e) was synthesized in our laboratories. NEIC and *n*-butylamine (99+%) were obtained from Aldrich (Milwaukee, WI, USA). Analytical-grade sodium carbonate, sodium hydroxide, sodium phosphate (dibasic), sodium chloride, HPLC-grade chloroform and hexane were obtained from Fisher Scientific (Fair Lawn, NJ, USA). Analytical-grade D-glucose, methanol and diethyl ether were obtained from BDH (Toronto, Canada). Water was double-distilled and filtered using a Millipore Milli Q filtration system (Mississauga, Canada). Bacto yeast extract was obtained from Difco Labs. (Detroit, MI, USA). *Cunninghamella echinulata* 4145 was obtained from the University of Alberta Microfungus Collection and Herbarium (Edmonton, Canada).

Chromatography

The HPLC system (Waters, Mississauga, Canada) consisted of a Model 590 pump, 712 WISP autosampler and 470 scanning fluorescence detector set at 280 and 340 nm for excitation and emission, respectively. Chromatographic data were collected using an NEC Powermate sx/16 computer (Boxborough, MA, USA). Peak integration was determined using the Baseline 810

program (Waters). Enantiomer separation was carried out on a 250 mm × 4.6 mm I.D. stainless-steel Partisil 5 column (Phenomenex, Torrance, CA, USA). The mobile phase consisted of hexane–chloroform–methanol (65:34:1, v/v) pumped at a programmed flow-rate of 0.8 ml/min for the first 12 min, and 2.5 ml/min for the remaining 23 min. Confirmation of identity of the mexiletine diastereomers was accomplished by comparing run times to similarly derivatized *R*-(−)-mexiletine and *S*-(+)-mexiletine standards.

Stock solutions

Separate 0.40 mg/ml solutions of (±)-mexiletine hydrochloride, (±)-HMM hydrochloride, (±)-PHM oxalate and (±)-prenalterol hydrochloride were prepared in distilled water and stored at 4°C. These were used to prepare working solutions containing 16, 32, 64, 100, 200 and 300 μg/ml mexiletine, HMM and PHM in microbial broth. A stock solution of NEIC (0.1%, v/v) was prepared in chloroform and stored under nitrogen at −10°C (solution 1). A solution of *n*-butylamine (0.33%, v/v, in chloroform, solution 2) was prepared prior to sample analysis. The microbial fermentation medium was prepared using distilled water, yeast extract (8.0 g/l), dibasic sodium phosphate (10 g/l) and sodium chloride (4.6 g/l). Medium pH was adjusted to 7.0, using 4 *M* sodium hydroxide, and autoclaved for 15 min. The D-glucose solution was prepared and autoclaved separately (final D-glucose concentration in the fermentation medium was 20 g/l). The complete medium was stored at −10°C.

Sample preparation

Suitable volumes of each working solution were diluted with distilled water to give final concentrations of 0.8, 1.6, 2.4, 5.0, 10.0 and 15.0 μg/ml of each racemate. Stock internal standard solution was added to each tube at a final concentration of 20 μg/ml. Aliquots of 0.25 ml were transferred to clean glass test tubes giving 0.2, 0.4, 0.8, 1.25, 2.5 and 3.75 μg of drug per tube. Sodium carbonate (0.100 ml of 0.2 *M* solution) and diethyl ether (2.0 ml) were added to each tube and these mixtures were vortex-mixed for 15 s and centrifuged at 1800 *g* for 4 min. The organic layer was removed and transferred to a clean glass test tube. The ether extraction step was repeated and the combined extracts were evaporated to dryness under a gentle stream of nitrogen. The residue was reconstituted in 0.3 ml of chloroform and derivatized with 0.075 ml of NEIC (solution 1) at room temperature. These solutions were vortexed for 10 s and again evaporated to dryness under a gentle stream of nitrogen. The residues were reconstituted with 0.220 ml of chloroform and reacted with 0.030 ml of *n*-butylamine (solution 2). Aliquots ranging from 0.01 to 0.065 ml were injected onto the HPLC system.

Extraction yield

A stock solution containing mexiletine, HMM and PHM was prepared in methanol (1.0 mg of each per ml). Suitable volumes of this stock solution were evaporated to dryness and reconstituted with 0.250 ml of diluted microbial broth (1:20) to give concentrations of 0.4 and 2.0 μg/ml of each compound per tube (six tubes of each). After addition of 0.1 ml of sodium carbonate (0.20 *M*), these solutions were extracted twice with diethyl ether (mixed for 15 s, centrifuged for 4 min). Exactly 1.5 ml from each extraction was transferred to a clean glass test tube and evaporated to dryness, using a gentle stream of nitrogen. Peak areas for each extraction of mexiletine, HMM and PHM were compared to unextracted peak areas of equivalent concentrations of drug under identical chromatographic conditions using a non-stereospecific, reversed-phase method [28].

Accuracy and precision

Racemic mexiletine, HMM and PHM were added to microbial broth ($n = 9$, three sets for three days) and the concentrations of individual enantiomers were calculated using a standard curve. Accuracy is defined as percent analytical recovery which is the mean result expressed as a percentage of the amount of analyte added (found/added × 100). Precision was determined by calculating the inter-assay coefficient of variation.

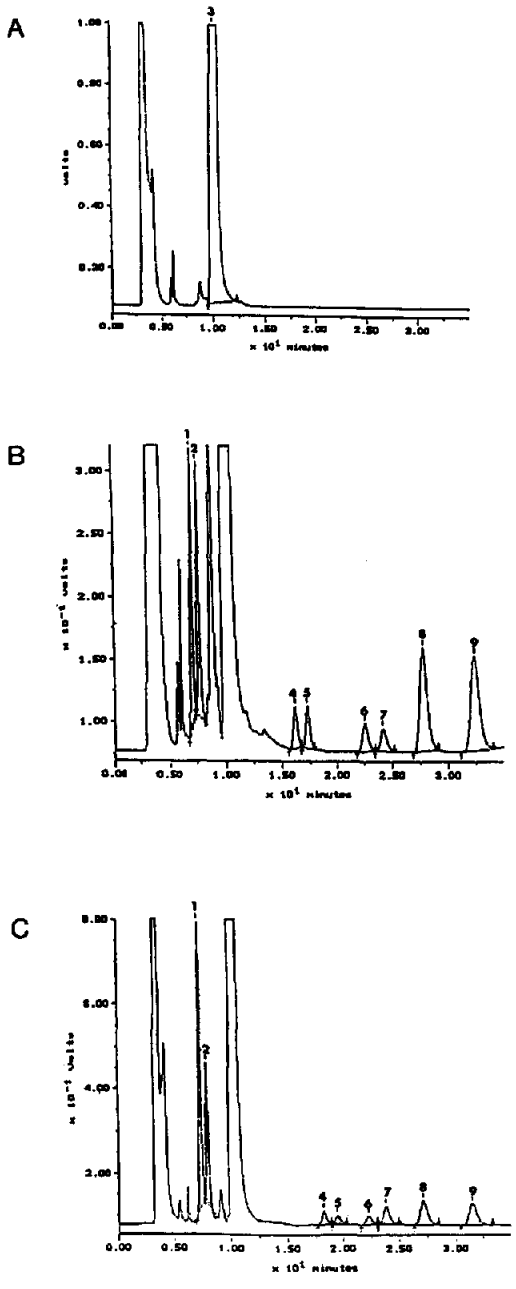

Fig. 2. Chromatograms of an extract from (A) blank microbial broth, (B) microbial broth spiked with 0.40 μg/ml mexiletine, HMM, PHM, and 4.0 μg/ml internal standard, (C) 72-h broth sample taken from a fermentation experiment. The fermentation vessel (125-ml Erlenmeyer flask) was inoculated with 300 μg/ml (±)-mexiletine at time 0. Peaks: 1 = *S*-(+)-mexiletine diastereomer; 2 = *R*-(−)-mexiletine diastereomer; 3 = the product of *n*-butylamine and reagent; 4 and 5 = HMM diastereomers; 6 and 7 = PHM diastereomers; 8 and 9 = internal standard diastereomers.

TABLE I

COMBINED EXTRACTION EFFICIENCY (MEAN OF SIX DETERMINATIONS)

Compound	Extraction yield (mean ± S.D., n = 6) (%)	
	0.4 μg/ml	2.0 μg/ml
Mexiletine	82.86 ± 5.32	72.81 ± 9.06
HMM	80.87 ± 13.52	80.78 ± 9.00
PHM	81.21 ± 7.01	70.85 ± 8.07

RESULTS AND DISCUSSION

Numerous analytical methods have been developed for the measurement of mexiletine alone [7,10–24,35–39] and for measuring mexiletine along with HMM and PHM [9,25–34]. To our knowledge, there have not been any reports of a stereospecific method capable of quantifying the enantiomers of all three compounds using a single procedure. Mexiletine, HMM and PHM all possess primary amine functional groups which can be derivatized with enantiopure isocyanates such as *S*-(+)-NEIC. This reagent is commercially available in high optical purity (99%). NEIC reacts with primary and secondary amines to form urea diastereomers which are sensitive to fluorescence detection. We have previously used this reagent to quantify the enantiomers of other cardiac drugs including (±)-acebutolol [40], (±)-tocainide [41], (±)-sotalol [42], and (±)-metoprolol [43].

Reaction of *S*-(+)-NEIC with (±)-mexiletine, (±)-HMM and (±)-PHM allowed the separation of all six diastereomers. Peaks corresponding to mexiletine eluted at 7.0 and 7.6 min with the first peak corresponding to *S*-(+)-mexiletine and the second to *R*-(−)-mexiletine. The order of elution was determined using optically pure *S*-(+)- and *R*-(−)-mexiletine standards derivatized under the same conditions. As we did not have access to optically pure standards of the metabolites and internal standard, we were unable to determine their elution order. Peaks corresponding

TABLE II

ACCURACY AND PRECISION OF THE METHOD (n = 9, THREE SETS FOR THREE DAYS)

Enantiomer amount added (μg)	Enantiomer amount measured (mean ± S.D.) (μg/ml)		Accuracy (analytical recovery, %)		Precision (C.V., %)	
	R	*S*	*R*	*S*	*R*	*S*
Mexiletine						
0.100	0.113 ± 0.010	0.114 ± 0.008	113.28	113.56	8.41	7.15
0.200	0.206 ± 0.012	0.209 ± 0.012	102.86	104.36	6.03	5.55
0.400	0.387 ± 0.010	0.389 ± 0.017	96.78	97.18	2.67	4.27
0.625	0.625 ± 0.028	0.628 ± 0.028	99.97	100.11	4.45	4.52
1.250	1.228 ± 0.040	1.228 ± 0.040	98.24	98.28	3.22	3.29
1.875	1.891 ± 0.023	1.891 ± 0.024	100.86	100.83	1.22	1.26
	Enantiomer amount measured (mean ± S.D.) (μg/ml)		Accuracy (analytical recovery, %)		Precision (C.V., %)	
	Peak 1	Peak 2	Peak 1	Peak 2	Peak 1	Peak 2
HMM						
0.100	0.108 ± 0.009	0.108 ± 0.009	107.88	107.69	8.21	8.38
0.200	0.213 ± 0.016	0.213 ± 0.017	106.36	106.53	7.69	7.84
0.400	0.398 ± 0.025	0.398 ± 0.025	99.40	99.42	6.21	6.29
0.625	0.598 ± 0.014	0.598 ± 0.015	95.72	95.75	2.36	2.50
1.250	1.231 ± 0.061	1.230 ± 0.061	98.48	98.41	4.98	4.95
1.875	1.895 ± 0.041	1.895 ± 0.041	101.06	101.09	2.19	2.16
PHM						
0.100	0.108 ± 0.008	0.108 ± 0.009	108.00	107.94	7.46	8.32
0.200	0.214 ± 0.008	0.215 ± 0.008	106.94	107.42	3.91	3.89
0.400	0.403 ± 0.008	0.403 ± 0.009	100.83	100.74	2.07	2.24
0.625	0.601 ± 0.019	0.601 ± 0.019	96.17	96.19	3.23	3.11
1.250	1.230 ± 0.031	1.229 ± 0.032	98.43	98.28	2.53	2.63
1.875	1.893 ± 0.018	1.895 ± 0.019	100.98	101.05	0.94	1.02

to the diastereomers of HMM and PHM eluted at 16.1 and 17.2 min and 22.5 and 24.1 min, respectively. Peaks corresponding to the diastereomers of the internal standard, (±)-prenalterol, eluted at 27.7 and 32.4 min. The peak eluting at 27.7 min was used in our calculations. Fig. 2 depicts a representative chromatogram of a broth blank (Fig. 2A), broth spiked with mexiletine (0.4 μg), metabolites (0.4 μg) and internal standard (20 μg) (Fig. 2B), and a broth sample taken from a microbial conversion experiment (Fig. 2C). This latter sample was taken 48 h after inoculating a fermentation vessel (125-ml Erlenmeyer flask) with 0.30 mg/ml (±)-mexiletine. All eight peaks were free from interference. Chromatographic resolution values (R_S) were 1.5 for mexiletine, 1.4 for HMM and PHM, and 2.2 for the internal standard.

Derivatization of antiarrhythmic agents and β-blockers with enantiopure isocyanates, under anhydrous conditions, is reported to be virtually complete after the reactants are evaporated to dryness [40,41,44,45]. The urea derivative is favored over the carbamate which is a consideration when derivatizing HMM, PHM and (±)-prenalterol. The hydroxyl group provides an ad-

ditional reaction site for excess NEIC. While this is not the preferred reaction, we have found that the hydroxyl group of (±)-prenalterol will slowly react to give the carbamate di-derivative. Therefore, reaction time should be controlled in order to ensure that the production of a di-derivative is minimized. This was accomplished by reacting excess NEIC with *n*-butylamine to give a derivative eluting at 10.1 min (Fig. 2A). This was confirmed by comparing a reagent blank with one containing only NEIC and *n*-butylamine.

The extraction efficiencies for mexiletine, HMM and PHM from microbial broth are summarized in Table I. This represents the total amount of drug removed from microbial broth, using a double extraction, which was required to achieve the desired sensitivity. A single extraction removed approximately 55% of each compound.

The assay is valid, as determined by precision (coefficient of variation) and accuracy (analytical recovery), for determination of the enantiomers of mexiletine, HMM and PHM (Table II). Good linearity ($r^2 > 0.99$) was observed over the entire concentration range examined. Calibration curves were described by $y = 0.067 + 0.78x$ for *S*-(+)-mexiletine, $y = 0.070 + 0.78x$ for *R*-(−)-mexiletine, $y = 0.19 + 2.85x$ and $y = 0.19 + 2.86x$ for the first and second peaks of HMM, respectively, and $y = 0.068 + 3.61x$ and $y = 0.073 + 3.82x$ for the first and second peaks of PHM, respectively.

In conclusion, the stereospecific procedure is capable of resolving the diastereomers of mexiletine, HMM, PHM and internal standard within 35 min using a normal-phase system. We were able to apply this method to the analysis of microbial broth for the biosynthesis of HMM and PHM from (±)-mexiletine, by *C. echinulata* (UAMH 4145). Our preliminary data (Fig. 2C) suggest that *C. echinulata* metabolizes (±)-mexiletine in a fashion similar to that observed in humans. In addition it appears that the mexiletine enantiomers are metabolized stereoselectively. This method is currently being evaluated for its ability to quantify the enantiomers of mexiletine and its metabolites in human serum.

ACKNOWLEDGEMENTS

We are grateful for the help we received from Cathy Lemko and Gordon Haverland in developing the methodology for this assay.

REFERENCES

1 B. J. Schrader and J. L. Bauman, *Drug Intell. Clin. Pharm.*, 20 (1986) 255.
2 P. B. Danneberg and J. H. Shelley, *Postgrad. Med. J. Suppl. 1*, 53 (1977) 25.
3 N. P. S. Campbell, J. G. Kelley, A. A. J. Adgey and R. G. Shanks, *Br. J. Clin. Pharmacol.*, 6 (1978) 103.
4 O. Grech-Bélanger, J. Turgeon and M. Gilbert, *Br. J. Clin. Pharmacol.*, 21 (1986) 481.
5 L. Igwemezie, C. R. Kerr and K. M. McErlane, *Xenobiotica*, 19 (1989) 677.
6 R. J. Hill, H. J. Duff and R. S. Sheldon, *Mol. Pharmacol.*, 34 (1988) 659.
7 J. Turgeon, A. C. G. Uprichard, P. M. Bélanger, D. W. G. Harron and O. Grech-Bélanger, *J. Pharm. Pharmacol.*, 43 (1991) 630.
8 L. F. Prescott, J. A. Clements and A. Pottage, *Postgrad. Med. J. Suppl. 1*, 53 (1977) 50.
9 A. H. Beckett and E. C. Chidomere, *J. Pharm. Pharmacol.*, 29 (1977) 281.
10 D. W. Holt, R. J. Flanagan, A. M. Hayler and M. Loizou, *J. Chromatogr.*, 169 (1979) 295.
11 K. J. Smith and P. J. Meffin, *J. Chromatogr.*, 181 (1980) 469.
12 J. Vasiliades, J. Kellett and R. S. Cox, *Am. J. Clin. Pathol.*, 81 (1984) 776.
13 D. F. LeGatt, R. J. Audette, O. Youzwishen and T. Stewart, *J. Pharm. Biomed. Anal.*, 9 (1991) 1147.
14 D. El Allaf, L. Henrard, L. Crochelet, D. Delapierre, J. Carlier and A. Dresse, *Br. J. Clin. Pharmacol.*, 14 (1982) 431.
15 H. Breithaupt and M. Wilfling, *J. Chromatogr.*, 230 (1982) 97.
16 O. Grech-Bélanger, J. Turgeon and M. Gilbert, *J. Chromatogr. Sci.*, 22 (1984) 490.
17 R. N. Gupta and M. Lew, *J. Chromatogr.*, 344 (1985) 221.
18 M. C. C. De Condado, A. Q. De Gainzarain, I. M. Mujica and J. A. C. Rodriguez, *J. Chromatogr.*, 530 (1990) 164.
19 N. Shibata, M. Akabane, T. Minouchi, T. Ono and H. Shimakawa, *J. Chromatogr.*, 566 (1991) 187.
20 A. Nishitani, S. Kanda and K. Imai, *Biomed. Chromatogr.*, 6 (1992) 124.
21 W. Mastropaolo, D. R. Holmes, M. J. Osborn, J. Rooke and T. P. Moyer, *Clin. Chem.*, 30 (1984) 319.
22 T. Pringle, J. Fox, J. A. McNeill, C. D. Kinney, J. Liddle, D. W. G. Harron and R. G. Shanks, *Br. J. Clin. Pharmacol.*, 21 (1986) 319.
23 F. Susanto, S. Humfeld and H. Reinauer, *Chromatographia*, 21 (1986) 41.

24 R. Verbesselt, T. B. Tjandramaga and P. J. De Schepper, *Ther. Drug Monit.*, 13 (1991) 157.
25 O. Grech-Bélanger, *J. Chromatogr.*, 309 (1984) 165.
26 O. Grech-Bélanger, J. Turgeon, M. Lalande and P. M. Bélanger, *Drug Metab. Dispos.*, 19 (1991) 458.
27 H. H. Mauer, *Arch. Toxicol.*, 64 (1990) 218.
28 N. A. Farid and S. M. White, *J. Chromatogr.*, 275 (1983) 458.
29 F. Broly, C. Libersa, M. Lhermitte and B. Dupuis, *Biochem. Pharmacol.*, 39 (1990) 1045.
30 K. Selinger and J. C. Crawhall, *J. Pharm. Biomed. Anal.*, 3 (1985) 547.
31 F. Broly, C. Libersa and M. Lhermitte, *J. Chromatogr.*, 431 (1988) 369.
32 M. Filipek, D. Paczkowski and J. Podlesny, *J. Chromatogr.*, 430 (1988) 406.
33 B. K. Krämer, K. M. Ress, F. Mayer, V. Kühlkamp, H. M. Liebich, T. Risler and L. Seipel, *J. Chromatogr.*, 493 (1989) 414.
34 M. Filipek, Z. Mielniczuk, J. Andrzejczak, W. Poplawska and D. Sitkiewicz, *J. Chromatogr.*, 573 (1992) 235.
35 O. Grech-Bélanger and J. Turgeon, *J. Chromatogr.*, 337 (1985) 172.
36 J. Gal, D. M. Desai and S. Meyer-Lehnert, *Chirality*, 2 (1990) 43.
37 Z. Abolfathi, P. Bélanger, M. Gilbert, J. R. Rouleau and J. Turgeon, *J. Chromatogr.*, 579 (1992) 366.
38 W. H. Pirkle and C. J. Welch, *J. Org. Chem.*, 49 (1984) 138.
39 K. M. McErlane, L. Igwemezie and C. R. Kerr, *J. Chromatogr.*, 415 (1987) 335.
40 M. Piquette-Miller, R. T. Foster, F. M. Pasutto and F. Jamali, *J. Chromatogr.*, 526 (1990) 129.
41 R. A. Carr, R. T. Foster, D. Freitag and F. M. Pasutto, *J. Chromatogr.*, 566 (1991) 155.
42 R. A. Carr, R. T. Foster and N. H. Bhanji, *Pharm. Res.*, 8 (1991) 1195.
43 M. M. Bhatti and R. T. Foster, *J. Chromatogr.*, 579 (1992) 361.
44 A. A. Gulaid, G. W. Houghton and A. R. Boobis, *J. Chromatogr.*, 318 (1985) 393.
45 P.-H. Hsyu and K. M. Giacomini, *J. Pharm. Sci.*, 75 (1986) 601.