Journal of Chromatography, 571 (1991) 169–177
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam

CHROMBIO. 6043

Reversed-phase liquid chromatographic method for the simultaneous determination of the antimalarial drugs sulfadoxine, pyrimethamine, mefloquine and its major carboxylic metabolite in plasma

YNGVE BERGQVIST*, SOLVEIG ECKERBOM, HELENA LARSSON and MONIREH MALEK-ZADEH

Department of Clinical Chemistry, Falun Central Hospital, S-791 82 Falun (Sweden)

(First received April 9th, 1991; revised manuscript received June 11th, 1991)

ABSTRACT

A high-performance liquid chromatographic (HPLC) method for the simultaneous determination of sulfadoxine, pyrimethamine, mefloquine and the carboxylic metabolite of mefloquine in plasma is described. After the proteins have been precipitated with a combination of zinc sulphate and acetonitrile containing two internal standards, pyrimethamine and mefloquine are extracted as bases and sulfadoxine and the carboxylic metabolite of mefloquine as ion-pairs with tetrabutylammonium. The drugs are separated by HPLC on a 3 μm octadecylsilica column with ultraviolet detection at 229 nm. The method is simple and reliable and enables the simultaneous determination of the drugs in 600-μl plasma samples with a sensitivity suitable for standard drug monitoring purposes.

INTRODUCTION

Fansimef (Hoffman-La Roche, Basle, Switzerland) is an antimalarial agent consisting of a fixed combination of pyrimethamine (P) (25 mg), mefloquine (MQ) (250 mg) and sulfadoxine (S) (500 mg) in a weight ratio of 1:10:20. Fansimef was formulated with the aim of delaying the development of *Plasmodium falciparum* multiresistant in the mid-1980s [1].

The mean plasma concentrations in adults during long-term malaria prophylaxis with one tablet of Fansimef once weekly were for P 0.4 μM, for S 190 μM and for MQ 2.3 μM [2]. In another prophylactic study with MQ once every second week, the mean plasma concentration of the carboxylic metabolite of MQ (MMQ) was 3.5 μM [3].

To evaluate the possible multidose interaction of S and P on the bioavailability of MQ, as well as inter-individual variability in the concentrations of the drugs, it may be clinically important to analyse the concentrations of the drugs. Several methods have been used to determine these compounds, including gas, thin-layer and high-performance liquid chromatography (HPLC), and some of them have

0378-4347/91/$03.50

been recently reviewed elsewhere [4]. Methods for the simultaneous determination of different antimalarial drugs, S and P [5], MQ and MMQ [6], P and MQ [7], MQ and chloroquine [8], MQ, S and P [9], have been presented. No method that enables simultaneous determination of S, P, MQ and MMQ has appeared so far.

This paper describes an HPLC method for the simultaneous determination of these compounds. Owing to the simultaneous extraction with ion-pair technique and subsequent simultaneous HPLC separation, the time required for each analysis is comparatively short.

EXPERIMENTAL

Chemicals

P, S, MQ and the acid metabolite of mefloquine [2,8-bis(trifluoromethyl)quinoline-4-carboxylic acid) were kindly supplied by Roche-Produkter (Skarholmen, Sweden). Sulfadimethoxine (SIS) from Sigma (St. Louis, MO, USA) and 2,8-bis(trifluoromethyl)-4-[1-hydroxy-3-(N-*tert.*-butylamino)propyl]quinoline phosphate (WR 184806) (IS) from Walter Reed Army Institute of Research (Washington DC, USA) were used as internal standards. Methyl *tert.*-butyl ether (MtBE) was obtained from Burdick and Jackson Labs. (Muskegon, MI, USA). Tetrabutylammonium (TBA) hydrogensulphate from Fluka (Buchs, Switzerland) and all other chemicals used (E. Merck, Darmstadt, Germany) were of analytical-reagent grade.

HPLC system

The HPLC system contained a pump, Model 2150 (LKB-Pharmacia, Uppsala, Sweden), and the sample was injected by use of a Waters WISP 710B injector, detected by a Waters UV 440 detector, with an extended-wavelength module monitored at 229 nm (Waters Assoc., Milford, MA, USA). For calculation of peak heights, a Model 4270 integrator from Spectra-Physics (San Jose, CA, USA) was used. The separation column was (150 mm × 4.0 mm I.D., packed with 3-μm particles of Spherisorb S3-ODS-1 (Hichrom, Reading, UK). The mobile phase was acetonitrile–0.1 *M* phosphate buffer (48:52, v/v), adjusted to pH 3.5. A flow-rate of 0.5 ml/min was used, giving a total retention time of *ca.* 20 min.

Comparison with previous HPLC methods

The method was validated by comparison with results of plasma drawn from individuals taking S, P or MQ for malaria prophylaxis and analysed by different HPLC methods [5,6].

Spiked plasma standards

Stock solutions were prepared at concentrations of MQ and MMQ at 100 μM, of P at [illegible] μM and of S at 10 m*M* in deionized water, and kept in polypropylene tubes. Standard samples were prepared by adding known amounts of the stock

solutions to plasma from healthy donors. The concentration ranges were: for S 1000–50 μM; for P 1–0.05 μM; for MQ 5–0.25 μM; and for MMQ 5–0.25 μM. A standard curve with five different concentrations was included in every assay run for calibration.

Internal standards

The internal standards (IS and SIS) were prepared at concentrations of 4 and 75 μM, respectively, in acetonitrile, used for protein precipitation.

Quality control

Quality control (QC) samples in spiked plasma containing S at 300 μM, P at 0.25 μM and MQ and MMQ at 3 μM were prepared and stored at −20°C for use in determining intra- and inter-assay precision and for the accuracy control during a study over a long period of time. The QC samples were analysed twice, at the beginning and at the end of each assay run.

Precision

The intra- and inter-assay precision of the method was determined by analysing twelve portions of three spiked plasma pools containing the drugs. The inter-assay precision was assessed by analysing one portion of each of three plasma pools on six different working days.

Recovery

The total extraction recovery of S, P, MQ, MMQ, SIS and IS was determined by comparing the peak heights of each component extracted from spiked samples with the peak heights obtained by direct injection of an aqueous solution containing the same concentrations of the drugs dissolved in the mobile phase.

Analysis of biological samples

To 600 μl of plasma or plasma standard in a 1.5-ml polypropylene tube, 150 μl of 0.1 M zinc sulphate were added during vortex-mixing for 15 s. Then 700 μl of acetonitrile with 4 μM IS and 75 μM SIS were added during vortex-mixing for 15 s. After 15 min the tubes were centrifuged at 10 000 g for 10 min. The supernatant was transferred to a new polypropylene tube. Then 2 ml of phosphate buffer (pH 9.0), 2 ml of 0.06 M TBA hydroxide and 5 ml of MtBE were added. The tube was shaken for 10 min and then centrifuged at 1200 g for 5 min. The upper organic layer was transferred to a conical polypropylene tube and evaporated to dryness at 50°C. The residue was reconstituted in 200 μl of mobile phase, and 100 μl were injected into the HPLC system.

RESULTS AND DISCUSSION

HPLC separation

We tested several C_{18} brands of column support (μBondapak, Waters; Ultrasphere ODS, Beckman; LiChrosorb RP-8, Merck), in order to achieve a separation of the compounds. The best separation results were achieved with 3 μm particles of Spherisorb S3-ODS-1 (Fig. 1). Other antimalarial drugs (proguanil, chloroquine, quinine, primaquine) have retention times different from S, P, MQ, MMQ and the internal standards did not interfere. The chosen mobile phase was acetonitrile–0.1 *M* phosphate buffer (48:52, v/v) adjusted to pH 3.5. The Spherisorb S3-ODS-1 column was remarkably rugged and stable. During the development of the assay more than 1000 plasma extracts were injected onto a single column. No significant deterioration of peak shape was noted over this period. The retention time variations obtained with different proportions of acetonitrile and phosphate buffer and at different pH values in 50% acetonitrile are shown in Fig. 2. The proportion of acetonitrile and, to a greater extent, the pH of the mobile phase, have a marked influence on the separation.

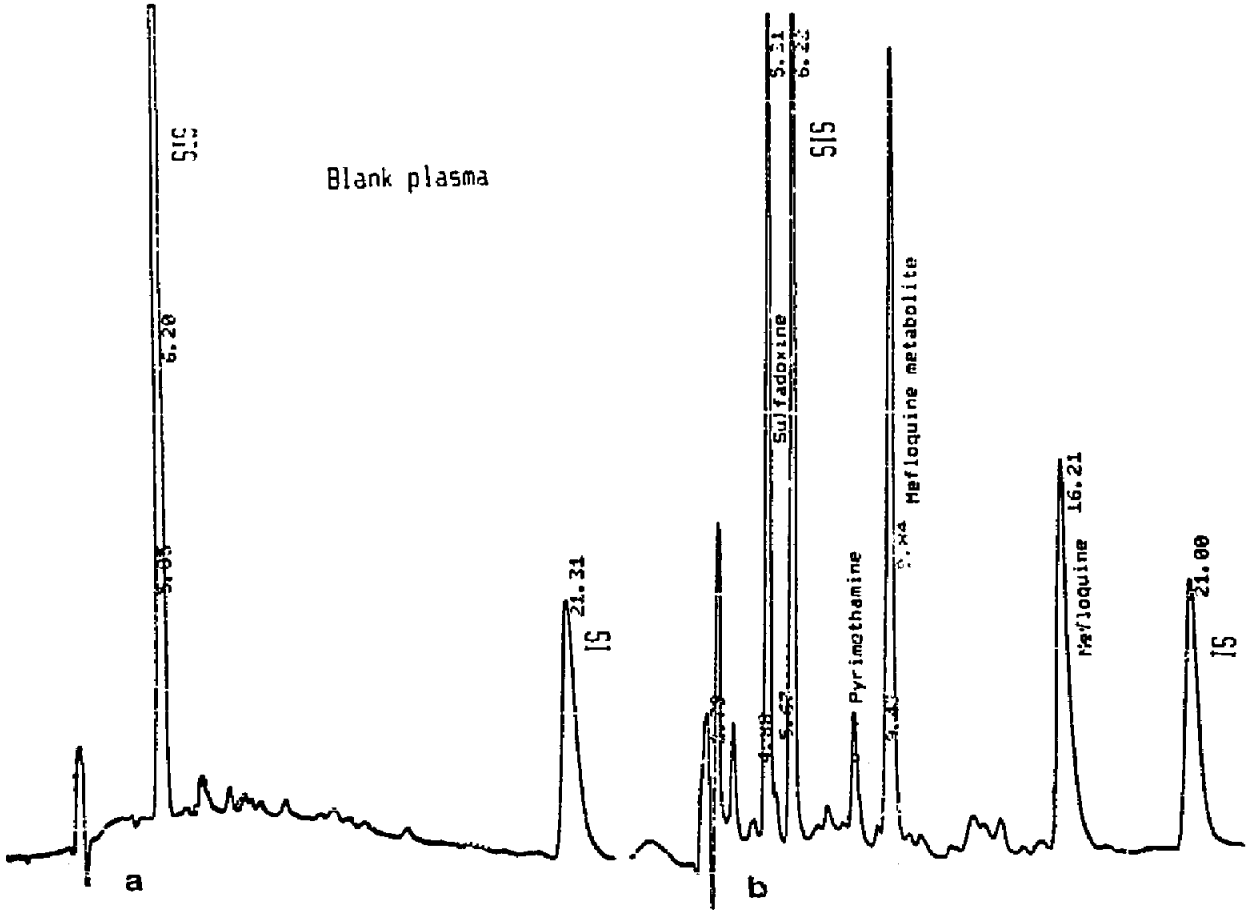

Fig. 1.

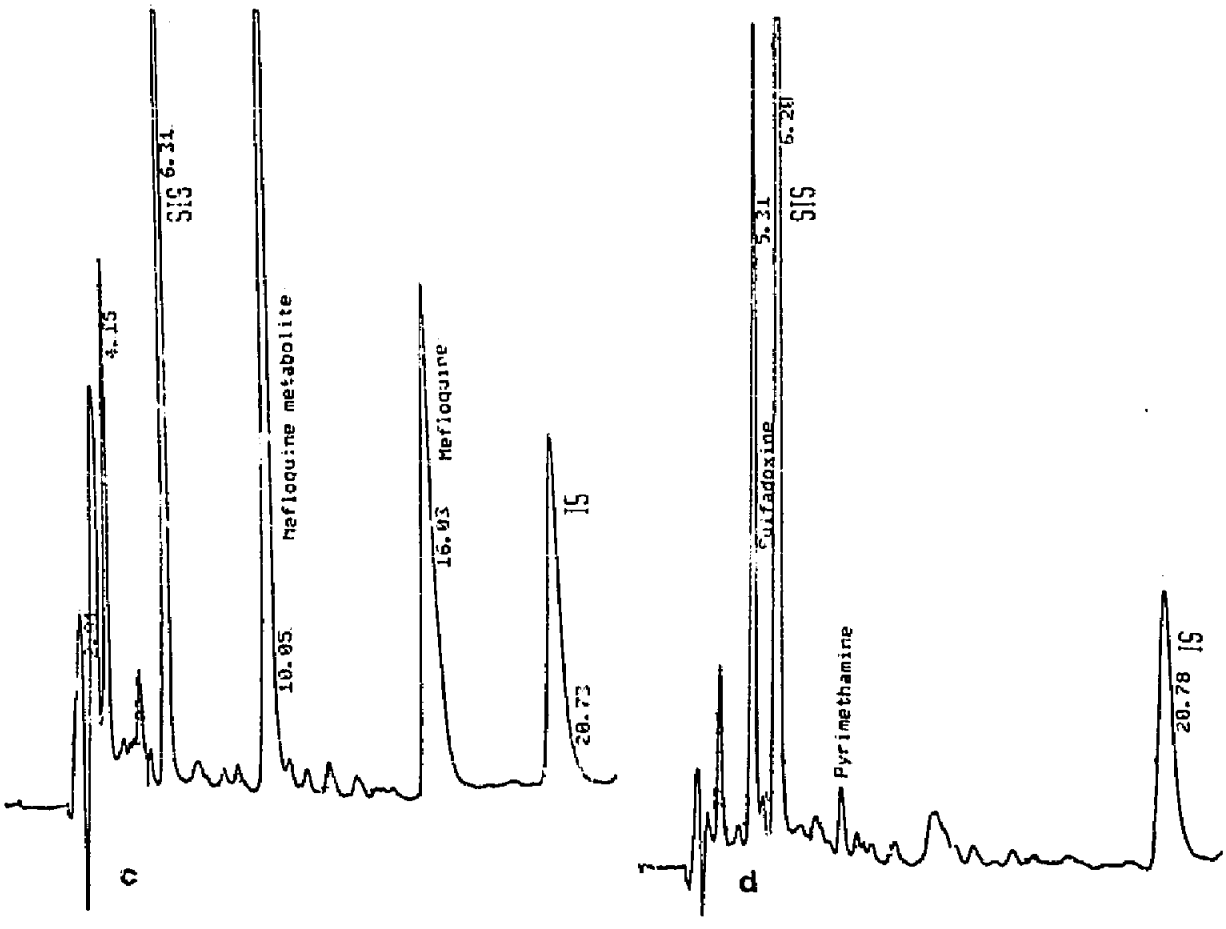

Fig. 1. Chromatograms of (a) human blank plasma, (b) plasma spiked with sulfadoxine (500 μM), pyrimethamine (0.50 μM), mefloquine (4.0 μM) and mefloquine metabolite (4.0 μM), (c) patient sample with mefloquine (4.8 μM) and mefloquine metabolite (5.5 μM), and (d) patient sample with sulfadoxine (650 μM), and pyrimethamine, (0.21 μM).

Extraction

Different extraction systems were evaluated in order to accomplish the simultaneous extraction of the drugs from biological material. MQ and P are basic compounds, and S and MMQ are acid compounds. Protein precipitation and subsequent ion-pair extraction has been used previously for simultaneous determination of MQ and MMQ in plasma and whole blood [6].

The highest recovery with a minimum of interference from endogenous compounds was achieved with a combination of protein precipitation and ion-pair extraction at pH 9.0 with 0.06 *M* TBA as the counter-ion and MtBE as the organic solvent. Higher concentrations of TBA did not improve the extraction efficiency for MMQ.

After protein precipitation, the basic compounds (MQ, P and IS) are extracted at pH 9.0 in uncharged form, by normal liquid–liquid extraction, and the acid

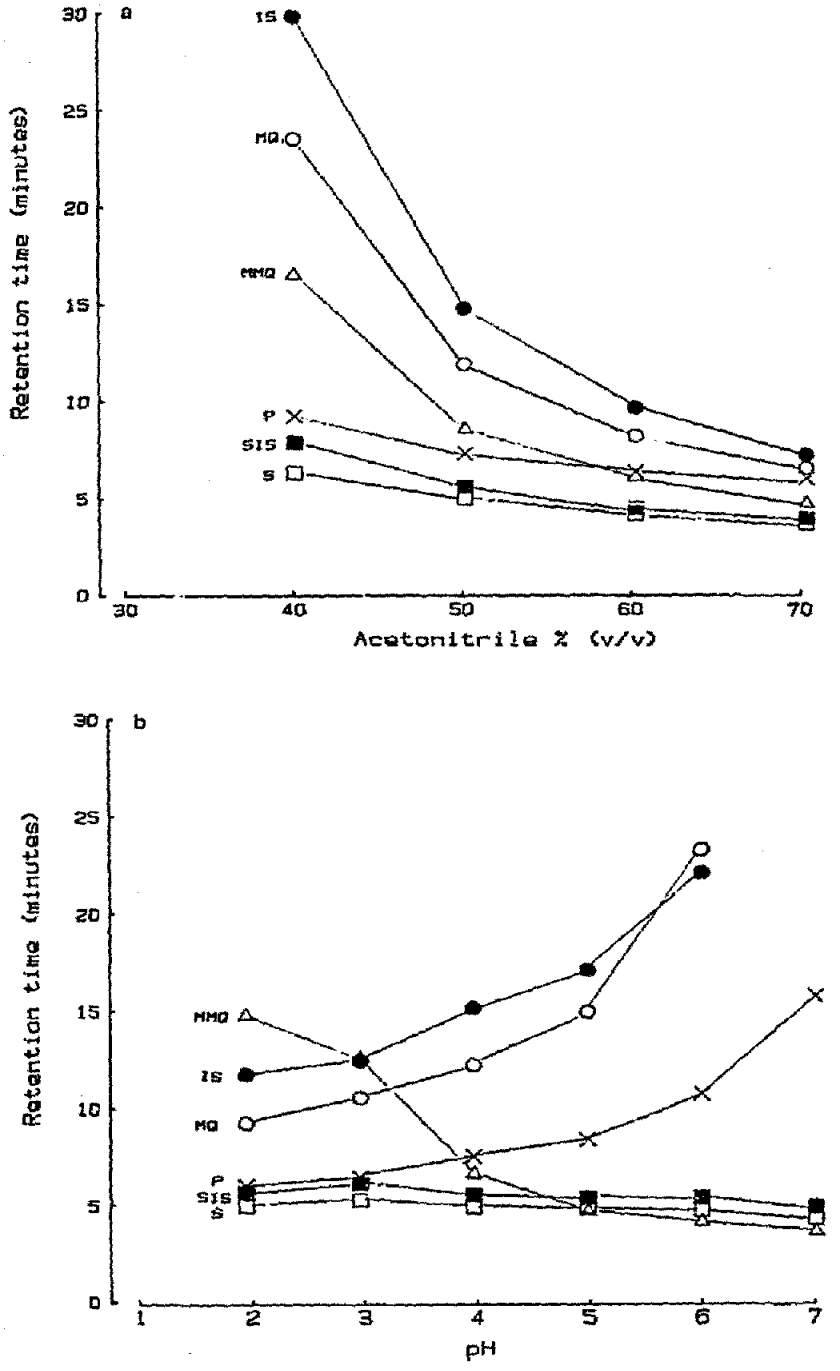

Fig. 2. (a) Effect of acetonitrile content of the mobile phase on the retention time in 0.1 *M* phosphate buffer (pH 3.5). (b) Effect of pH of the mobile phase on the retention time in 45% (v/v) acetonitrile.

compound (MMQ, S and SIS) in charged form as ion pairs with TBA. The extraction recoveries are presented in Table I. MtBE is a very good extraction solvent for MQ and MMQ and it gives a low endogenous background [6].

TABLE I

RECOVERY DATA

The total recovery from the extraction was tested by comparison with the peak heights obtained by direct injection of known amounts of the substances into the column.

Drug	Concentration (μM)	Recovery (mean ± S.D.) (%)	n
Sulfadoxine	500	1.92 ± 0.21	5
Pyrimethamine	0.5	74.6 ± 1.8	7
Mefloquine	5	76.5 ± 8.0	5
	1.25	84.9 ± 8.6	5
Mefloquine metabolite	5	63.4 ± 4.8	8
	1.25	73.1 ± 3.2	5
Internal standard (SIS)	75	6.28 ± 0.56	6
Internal standard (IS)	4	86.4 ± 4.9	5

Limit of determination

The limit of determination of the drugs was defined as the minimum concentration that gave an interassay coefficient of variation (C.V.) of less than 15%. By this definition, and using 600 μl of plasma, the limits were estimated to 75, 0.050, 0.25 and 0.25 μM for S, P, MQ and MMQ, respectively.

Comparison with previous HPLC methods

The results from the comparison are shown in Table II.

Recovery and precision

The total recovery from plasma for S was only 2%, which is similar to that of the internal standard (SIS). However, there is sufficient S in plasma during malaria prophylaxis for reliable quantitation in the chromatographic system. The addition of the extra internal standard (SIS) specially for S yielded an improvement in the precision for S, as is shown in Table III. The low recovery depended on the

TABLE II

COMPARISON WITH OTHER HPLC METHODS

Drug and ref.	Number of samples	Correlation coefficient	Regression equation
MQ [5]	18	0.99	$y = 1.034x - 0.040$
MMQ [5]	18	0.98	$y = 0.965x + 0.026$
S [6]	18	0.95	$y = 1.029x - 9.47$
P [6]	15	0.98	$y = 0.911x - 0.024$

TABLE III

PRECISION DATA

Spiked plasma sample; $n = 6$.

Drug	Concentration (μM)	Relative standard deviation (%)	
		Intra-assay	Inter-assay
Sulfadoxine	1000	1.96	3.93
	500	2.31	6.12
	250	2.22	5.28
	125	11.3	13.3
	63	11.9	15.6
Pyrimethamine	1.0	3.40	3.50
	0.25	4.77	7.25
	0.060	5.71	7.79
Mefloquine	5.0	2.32	8.55
	1.25	2.70	4.63
	0.32	6.44	9.02
Mefloquine metabolite	5.0	2.78	4.24
	1.25	1.39	9.10
	0.32	3.76	9.29

fact that 0.06 *M* TBA forms an ion pair with S and SIS with very low extraction at pH 9.0 in MtBE. Decreasing the pH of the buffer during the extraction results in a significant improvement of the extraction efficiency for S and SIS. In addition, it resulted in a very large chromatographic peak for S with low separation efficiency between S and SIS. The recovery of P, MQ and MMQ from the same spiked plasma samples ranged from 63 to 85%. The reproducibilities within and between days are shown in Table III, and demonstrate a good precision of the method over the therapeutic concentration range.

CONCLUSION

The therapeutic plasma concentration of P is much lower than those of the other drugs, therefore the chromatographic and extraction procedures were optimized for P, under acceptable recovery and precision conditions for the other drugs. A high concentration of S in plasma in relation to the other drugs, in combination with a low recovery and a detection wavelength with a low absorptivity for S, results in similar peak heights in the liquid chromatogram for S as for the other compounds in plasma samples obtained during malaria prophylaxis.

The limits of determination and the precisions of the present method are adequate for monitoring the drug concentrations in human plasma during regular propylaxis with Fansimef, and the method is comparatively rapid, simple, reliable and sensitive.

ACKNOWLEDGEMENTS

We are grateful to Dr. Urban Hellgren and Dr. Lars Rombo for valuable discussions and their interest in his work.

REFERENCES

1 B. Merkli and R. Richle, *Ann. Trop. Med. Parasitol.*, 78 (1984) 459.
2 D. E. Schwartz, E. Weidekamm, I. Mimica, P. Heizmann and R. Portmann, *Chemotherapy*, 33 (1987) 1.
3 U. Hellgren, V. H. Angel, Y. Bergqvist, A. Arvidsson, J. S. Forero-Gomez and L. Rombo, *Trans. R. Soc. Trop. Med. Hyg.*, 84 (1990) 46.
4 Y. Bergqvist and F. C. Churchill, *J. Chromatogr.*, 434 (1988) 1.
5 Y. Bergqvist and M. Eriksson, *Trans. R. Soc. Trop. Med. Hyg.*, 79 (1985) 297.
6 Y. Bergqvist, U. Hellgren and F. C. Churchill, *J. Chromatogr.*, 432 (1988) 253.
7 A. Guenzi, G. Cappellett, A. Scala and M. Zanetti, *J. Chromatogr.*, 494 (1989) 219.
8 J. M. Lamant, P. Kintz, A. Tracqui, P. Mangin, A. A. Lugnier and A. J. Chaumont, *Ann. Biol. Clin.*, 46 (1988) 722.
9 M. D. Edstein, I. D. Lika, T. Chongsuphajaisiddhi, A. Subcharcon and H. K. Webster, *Ther. Drug Monit.*, 13 (1991) 146.