Journal of Chromatography, 430 (1988) 351–360
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 4273

DETERMINATION OF PEMOLINE IN PLASMA, URINE AND TISSUES BY REVERSED-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

TAKAO AOYAMA*, HAJIME KOTAKI, YUKIYA SAITOH and FUJIO NAKAGAWA

Hospital Pharmacy, Faculty of Medicine, University of Tokyo, 3-1, Hongo 7-chome, Bunkyo-ku, Tokyo 113 (Japan)

(First received December 29th, 1987; revised manuscript received April 22nd, 1988)

SUMMARY

A simple and selective high-performance liquid chromatographic method with ultraviolet detection at 215 nm for the determination for pemoline in rat plasma, urine and tissues is described. Pemoline in the samples was extracted with methylene chloride at pH 10 and the organic phase was evaporated after adding 5-methyl-5-phenylhydantoin used as an internal standard. Pemoline and the internal standard were separated on a Kaseisorb LC C8-60-5 reversed-phase column. The limits of determination of pemoline in 0.1–0.2 ml of plasma, urine and tissue homogenates were 2, 100 and 20 ng, respectively. The method should be useful for studies of the pharmacokinetics and distribution of pemoline in small animals.

INTRODUCTION

Pemoline (2-amino-5-phenyl-2-oxazolin-4-one; the structural formula is shown in Fig. 1) is a central nervous system stimulant and has been used as a drug for the treatment of mild depression [1], narcolepsy [2] and attention deficit disorder with hyperactivity [3–5].

Several procedures for the determination of pemoline in biological fluids have been developed. However, an earlier spectrophotometric method [6] and radiometric methods [7,8] suffer from a lack of sensitivity and/or selectivity. Gas chromatographic (GC) methods [9–16] have been reported for the determination of pemoline in the plasma, serum, saliva and urine of man or horses. Most of these methods were based on conversion from pemoline to an acidic hydrolysis product, 5-phenyl-2,4-oxazolidinedione, followed by derivatization. Large volumes (0.3–10 ml) of biological fluid samples were required. A gas chromatographic–mass spectrometric method [17,18] also involved the same procedures

0378-4347/88/$03.50

as those in the GC methods as above. High-performance liquid chromatographic (HPLC) methods [3–5,19–21] using an ultraviolet detector have been reported for the determination of pemoline in the serum, plasma, saliva and urine of man. Most of these HPLC methods were based on direct extraction of pemoline into an organic solvent from the biological samples [3–5] or deproteinization with acetonitrile [21]. These methods have relatively high sensitivity, and the volume of plasma sample used was small (0.1 ml).

Several studies on the pharmacokinetics of pemoline in man [3–5,11,13,21] and animals [17] have been reported. However, there is little information on the tissue distribution of pemoline. For detailed studies of the pharmacokinetics and distribution of pemoline in small laboratory animals such as rats, it is necessary to have a reliable microanalytical method for determining concentrations of pemoline in tissues in addition to plasma and urine.

In this paper, we describe a simple and selective HPLC method for the determination of pemoline in plasma, urine and tissues.

EXPERIMENTAL

Materials and reagents

Pemoline was kindly supplied by Sanwa Kagaku Kenkyusho (Nagoya, Japan). 5-Methyl-5-phenylhydantoin used as an internal standard (I.S.) (the structural formula is shown in Fig 1) was obtained from Aldrich (Milwaukee, WI, U.S.A.). 5-Phenyl-2,4-oxazolidinedione, a minor metabolite of pemoline, was synthesized by hydrolysing pemoline in solution acidified with sulphuric acid according to the method of Libeer and Schepens [9]. White crystals were obtained with a melting point of 107°C (lit. [9], 107°C). Acetonitrile was of HPLC grade. All other solvents and reagents were of analytical-reagent grade.

Apparatus and HPLC conditions

An LC-6A liquid chromatograph and an SPD-6A spectrophotometer (Shimadzu Seisakusyo, Kyoto, Japan) were used. The ultraviolet wavelength of the detector was set at 215 nm. The reversed-phase column was a stainless-steel tube (250 mm×4 mm I.D.) packed with Kaseisorb LC C8-60-5, particle size 5 μm (Tokyo Kasei Kogyo, Tokyo, Japan). The column temperature was maintained at 28°C by a column jacket in which thermostatically controlled water was circulating. The mobile phase was distilled water (pH 5, adjusted by dropwise addition of 0.015 *M* phosphoric acid)–acetonitrile (80:20, v/v). The mobile phase was degassed before use. The flow-rate of mobile phase was 0.7 ml/min.

Extraction procedure

Plasma. To 0.1 ml of rat plasma in a 10-ml glass-stoppered centrifuge tube were added 0.5 ml of 1 *M* carbonate buffer solution (pH 10) and 4 ml of methylene chloride. The mixture was shaken for 10 min and centrifuged at 1680 *g* for 5 min. After the upper aqueous phase had been removed by aspiration, 3 ml of the organic phase were transferred into another centrifuge tube and 1 ml of methylene chloride containing 1 μg/ml I.S. was added. The mixture was evaporated to dry-

ness with a rotary evaporator at room temperature, the residue was dissolved in 50 μl of mobile phase and 20-μl aliquots of this solution were injected into the HPLC column.

Urine. Extraction of pemoline from 0.1 ml of rat urine was carried out by the same manner as above, up to removal of the aqueous phase. Then 1 ml of the organic phase was transferred into another centrifuge tube and 1 ml of methylene chloride containing 4 μg/ml I.S. was added. After evaporation to dryness, the residue was dissolved in 50 μl of mobile phase and 5-μl aliquots of this solution were injected into the HPLC column.

Tissues. Brain, liver, kidney, lung and spleen samples were homogenized on ice with 2 volumes of ice-cold normal saline solution and muscle samples with 3 volumes of the same solution.

Liver, kidney, lung, spleen and muscle homogenates. To 0.2 ml of each tissue homogenate were added 0.5 ml of 1 *M* carbonate buffer solution (pH 10) and 4 ml of methylene chloride. The mixture was shaken and centrifuged as above. After removal of the aqueous phase, 3 ml of the organic phase were transferred into another centrifuge tube and 1 ml of methylene chloride containing 1 μg/ml I.S. was added. The resulting mixture was processed according to the procedure for plasma.

Brain homogenate. The extraction of pemoline from 0.2 ml of the homogenate and subsequent evaporation to dryness after addition of the I.S. were as described above. The residue was dissolved in 50 μl of mobile phase and the solution was loaded into a capillary tube (Drummond Microdispenser; Drummond Scientific, U.S.A.) sealed at one end by heating and was centrifuged at 15 300 *g* for 5 min. Then 20-μl aliquots of the supernatant were injected into the HPLC column.

Calibration graphs

A series of solutions containing various concentrations of pemoline were prepared by dissolving the drug in methanol (20, 50, 200, 1000, 2000, 4000 and 10 000 ng/ml of pemoline for plasma, 1, 4, 10, 25, 50, 100, 250 and 500 μg/ml for urine and 200, 400, 800, 1600 and 3200 ng/g for tissues), and 0.1 ml of each solution was placed in a centrifuge tube. The solvent was evaporated and to each residue were added 0.1 ml of plasma, 0.1 ml of urine or 0.2 ml of tissue homogenate. These samples were then assayed by the method described for the extraction procedure.

The peak-height ratios were calculated by dividing the peak height of pemoline by that of the I.S. and were plotted against the amount of pemoline.

Recoveries

The extraction recoveries of pemoline from each biological sample were examined for two different amounts of pemoline. Plasma samples containing 20 and 100 ng, urine samples containing 1 and 10 μg and brain, liver and muscle homogenate samples containing 20 and 100 ng of pemoline were prepared as described for the calibration graphs. The peak-height ratios were compared with those obtained by direct assay of pemoline standards equivalent to a 100% extraction yield in the extraction step.

Stabilities in alkaline solutions

Stability in 1 M carbonate buffer solution (pH 10). A 10-ml volume of 1 *M* carbonate buffer solution (pH 10) containing 4 μg/ml pemoline was incubated at 26°C and 60, 120, 180 and 240 min after starting the incubation 0.5 ml of the solution was removed. Immediately, the pH of these sample solutions was adjusted to 7.2 by adding 2 ml of 0.5 *M* phosphate buffer solution (pH 6.3) to terminate the degradation of the drug. For analysis, 0.2 ml of the mixture was used.

Stability in 0.04 M sodium hydroxide solution. Pemoline (60 mg) was dissolved in 30 ml of ice-cold 0.04 *M* sodium hydroxide solution by sonication. Three 9-ml aliquots from the solution were incubated at 6–7, 26 and 37°C and 10, 20, 30, 45, 60, 90 and 120 min after starting the incubation 0.2 ml of the solutions was removed. Immediately, the pH of the sample solutions was adjusted to 7.4 by adding 5 ml of 1/15 *M* phosphate buffer solution (pH 7.4) to terminate the degradation of the drug. For analysis, 0.2 ml of the mixture was used.

The addition of the phosphate buffer solution mentioned above is based on our finding that pemoline was stable for at least 48 h when the pH of the solution was adjusted to 7.2–7.4.

Drug administration and sample collection

Adult male Wistar rats (270–300 g) were used. A solution of pemoline for injection was prepared by dissolution in 0.04 *M* sodium hydroxide solution. A dose of 2 mg/kg of pemoline was administered intravenously to rats. The rats were killed 0.25, 1, 3, 5 and 10 h after administration by forced bleeding via a cannula inserted into a femoral artery, and brain, liver, kidney, lung, spleen and muscle samples were excised quickly. Plasma was separated by centrifugation. In another experiment, urine was collected via a cannula inserted into the bladder for 24 h after intravenous administration of the same dose of pemoline. Plasma, urine and tissue samples were stored at −20°C until analysis.

RESULTS AND DISCUSSION

Extraction and chromatographic conditions

It was reported that pemoline is a weak acid with a pK_a of 10.5 [14] and is extracted into organic solvents over a wide pH (0.5–11) [19]. In preliminary tests with 0.5 *M* acetate (pH 5), 0.5 *M* phosphate (pH 7) and 0.5 *M* carbonate (pH 10) buffer solutions, the last was the most suitable buffer solution for addition to the biological sample, as it produced the smallest endogenous peak on the chromatogram under the HPLC conditions used. In the examination of the recovery of pemoline from rat plasma, when 0.5 ml of 1, 0.5 and 0.1 *M* carbonate buffer solution (pH 10) was added to plasma containing 200 ng of pemoline and the drug was extracted with methylene chloride, the mean recoveries of pemoline were 60, 51 and 40%, respectively. From this finding, 1 *M* carbonate buffer solution was selected for addition to the biological samples. Further, with the use of 0.1 ml of 1 *M* carbonate buffer solution (pH 10), the mean recovery was 77%. However, larger endogenous peaks were produced. Therefore, 0.5 ml of buffer solution were added to the biological samples.

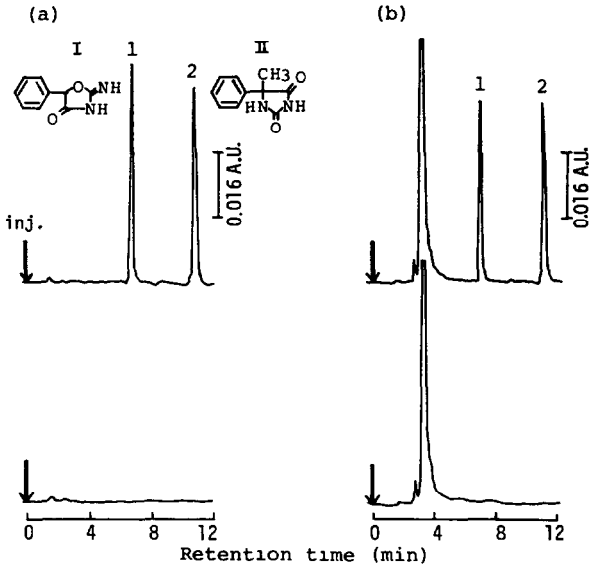

Fig. 1. Chromatograms of pemoline and 5-methyl-5-phenylhydantoin (I.S.) from: (a) 0.1 ml of plasma containing 300 ng of pemoline (upper trace) and 0.1 ml of drug-free plasma (lower trace); (b) 0.1 ml of urine containing 3 μg of pemoline (upper trace) and 0.1 ml of drug-free urine (lower trace). Known amounts of I.S. were added after extraction as described in the extraction procedure. Peaks: 1 = pemoline; 2 = I.S. Structural formulae: I = pemoline; II = I.S.

For brain, a clean-up step for separation of biological components at 15 300 g was necessary, as the centrifugation at 1680 g after the extraction was not adequate. The reason for the addition of the I.S. after completion of the extraction was that the recovery of the I.S. from the biological samples was relatively low (less than 50%) and fluctuated [coefficient of variation (C.V.) less than 15%, $n=5$] under the HPLC conditions used. A UV wavelength at 215 nm was used to determine pemoline, as absorption maximum was observed at 215 nm and the molar absorptivity was $2.6 \cdot 10^4$ mol^{-1} cm^{-1}.

Typical chromatograms of pemoline and the I.S. obtained from plasma and urine samples are shown in Fig. 1 and those of pemoline and the I.S. obtained from brain, liver and muscle samples are shown in Fig. 2. The peaks of pemoline and the I.S. in each chromatogram were sharp and well separated from the endogenous peaks, and their retention times were approximately 7 and 11 min, respectively.

It has been reported that 5-phenyl-2,4-oxazolidinedione, a minor metabolite of pemoline, was not detected in man, rat and dog but was detected in rabbit [8]. Although the retention time of the metabolite was approximately 21 min with direct injection on to the HPLC column, we confirmed that the metabolite was hardly extracted under the conditions used.

Calibration graphs, recoveries and reproducibilities

Linear relationships were found between the peak-height ratio and the amount of pemoline in the ranges 2–1000 ng for 0.1 ml of plasma, 0.1–50 μg for 0.1 ml of

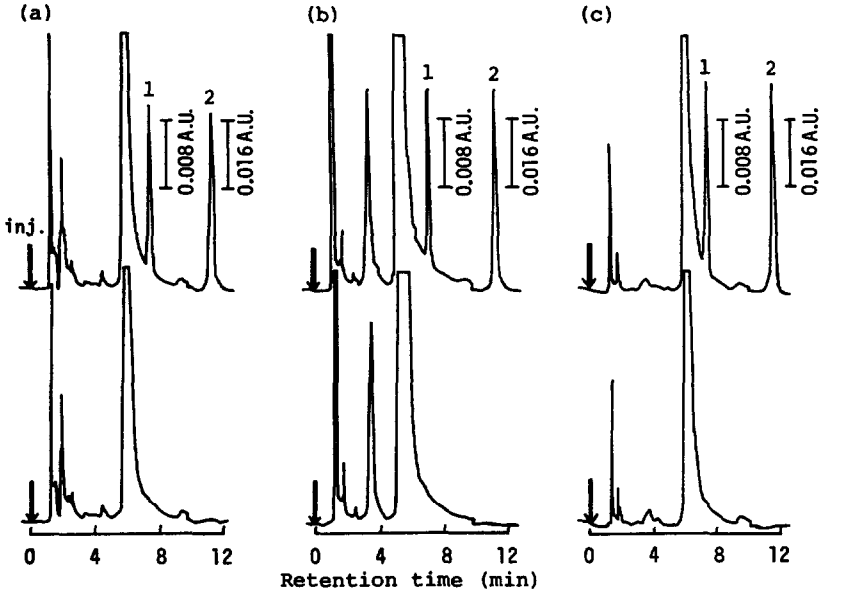

Fig. 2. Chromatograms of pemoline and 5-methyl-5-phenylhydantoin (I.S.) from: (a) 0.2 ml of homogenate of brain containing 150 ng of pemoline (upper trace) and 0.2 ml of drug-free homogenate of brain (lower trace); (b) 0.2 ml of homogenate of liver containing 150 ng of pemoline (upper trace) and 0.2 ml of drug-free homogenate of liver (lower trace); (c) 0.2 ml of homogenate of muscle containing 150 ng of pemoline (upper trace) and 0.2 ml of drug-free homogenate of muscle (lower trace). Known amounts of I.S. were added after extraction as described in the extraction procedure. Peaks: 1 = pemoline; 2 = I.S.

urine and 20–320 ng for 0.2 ml of brain, liver and muscle, as given by the equations $y = 0.00267x - 0.001$, $y = 0.225x - 0.013$, $y = 0.00242x - 0.005$, $y = 0.00235x - 0.010$ and $y = 0.00254x - 0.003$, respectively. The calibration graphs for kidney, lung and spleen samples were almost identical with that for the liver homogenate. The limits of detection of pemoline were 0.5 ng for plasma, 5 ng for urine and 2 ng for tissue homogenates with a signal-to-noise ratio of ca. 10:1.

The extraction recoveries of pemoline from plasma, urine and tissue samples prepared by adding known amounts of the drug are shown in Table I. The coefficients of variation of the recovery obtained by repeating the procedure three or four times for each sample were less than 4.1% and the reproducibilities were good. Table II shows the within- and between-day precisions for plasma and liver samples. Similar results were obtained with urine, brain, kidney, lung, spleen and muscle samples.

Stability of pemoline in alkaline solution

It has been reported that pemoline was hydrolysed to mandelic acid in strongly alkaline solution [12]. Therefore, the stabilities of pemoline in 1 *M* carbonate buffer solution (pH 10) as used in the extraction procedure and in 0.04 *M* sodium hydroxide solution (pH $\approx$12) as used as the dissolution agent for administration of pemoline to rats were examined.

As shown in Table III, in 1 *M* carbonate buffer solution no degradation of

TABLE I

RECOVERIES OF PEMOLINE FROM RAT PLASMA, URINE AND TISSUES AND THEIR REPRODUCIBILITIES

Sample	Amount added	Recovery* (mean ± S.D.) (%)	C.V.* (%)
Plasma	20 ng	60.3 ± 1.2	2.0
	100 ng	60.1 ± 1.0	1.7
		Mean: 60.2	
Urine	1 μg	62.2 ± 2.2	3.5
	10 μg	62.0 ± 1.9	3.1
		Mean: 62.1	
Brain	20 ng	55.8 ± 2.3	4.1
	100 ng	54.4 ± 0.4	0.7
		Mean: 55.2	
Liver	20 ng	52.0 ± 0.9	1.8
	100 ng	51.1 ± 0.3	0.6
		Mean: 51.6	
Muscle	20 ng	55.9 ± 0.8	1.4
	100 ng	54.9 ± 0.4	0.6
		Mean: 55.4	

*Mean ± standard deviation and coefficient of variation for three or four determinations.

TABLE II

PRECISION OF THE ASSAY OF PEMOLINE IN PLASMA AND LIVER SAMPLES

Means ± standard deviations and coefficients of variation for four determinations.

Sample	Concentration found (mean ± S.D.) (ng/ml or ng/g)	C.V. (%)
Within-day		
Plasma	2190 ± 35	1.6
	268 ± 5	2.0
Liver	2992 ± 42	1.4
	392 ± 13	3.4
Between-day		
Plasma	2117 ± 44	2.1
	266 ± 6	2.5
Liver	2808 ± 53	1.9
	401 ± 12	3.0

pemoline was observed at 26°C, at least up to 120 min. Therefore, it is not necessary to consider the degradation of pemoline in the extraction step when the drug is extracted immediately from biological samples with an organic solvent after adding the buffer solution.

TABLE III

EFFECTS OF TEMPERATURE ON DEGRADATION OF PEMOLINE IN TWO DIFFERENT ALKALINE SOLUTIONS

Time (min)	Residual concentration of pemoline (%)			
	1 *M* carbonate buffer solution (pH 10) at 26°C*	0.04 *M* sodium hydroxide solution**		
		6–7°C	26°C	37°C
0	100.0	100.0	100.0	100.0
10	–	101.7	99.5	–
20	–	100.7	99.6	–
30	–	102.0	99.7	92.9
45	–	101.5	98.8	–
60	100.2	99.5	97.5	84.7
90	–	97.1	91.3	–
120	99.9	97.9	90.6	74.8
180	96.8	–	–	–
240	95.3	–	–	–

*Initial concentration of pemoline in the solution, 4 μg/ml.
**Initial concentration of pemoline in the solution, 2 mg/ml; pH of the solution, ca. 12.

The stability of pemoline in 0.04 *M* sodium hydroxide solution was examined at three temperatures (Table III). When the alkaline solution containing pemoline was incubated at 37°C, pemoline was gradually degraded and the residual concentration of the drug was 70% at 120 min. However, no degradation of pemoline at 6–7°C was observed, at least up to 60 min. At 26°C, no degradation of the drug was observed up to 30 min, and the residual concentration was 97.5% even 60 min later. From these results, it was concluded that the degradation of the drug in alkaline solution could be prevented if the solution was used within 1 h at temperatures below 26°C. In addition, severe haemolysis and haematuria were not observed with the naked eye when an alkaline solution (dosing volume 1 ml/kg) was administered intravenously to rats.

Application of the method

The method described was applied to the determination of pemoline concentrations in rat plasma, urine and tissues. Concentrations of pemoline in plasma and several tissues 0.25, 1, 3, 5 and 10 h after intravenous administration of pemoline at a dose of 2 mg/kg are shown in Fig. 3. The concentrations of pemoline in the liver, kidney, lung, spleen and muscle decreased with time in relation to that in plasma. Among these tissues, the highest concentrations of pemoline were found in the kidney, followed by the liver. In the brain, it was found that the concentration increased gradually up to 1 h after administration and thereafter decreased in proportion to that in plasma. A mean of 60% of the dose was recovered in urine as unchanged pemoline after 24 h.

As shown in Fig 3, it is interesting that the points for plasma concentration plotted on semilogarithmic graph paper tended to curve inwards. Studies on this

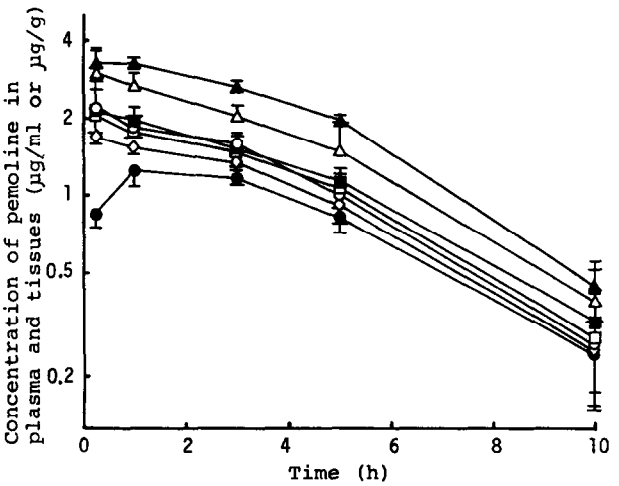

Fig. 3. Time courses of plasma and tissue concentrations of pemoline in rats after intravenous administration at a dose of 2 mg/kg body mass of pemoline. Each point represents the mean of three or four determinations and the vertical bar indicates the standard deviation. ○, Plasma; ●, brain; △, liver; ▲, kidney; ■, lung; □, spleen; ◇, muscle.

non-linear disposition of the drug in rats are in progress using the method described here. These results will be reported elsewhere.

In conclusion, we have established a simple and selective HPLC method for the determination of pemoline in plasma, urine and tissues. Pemoline in 0.1 ml of plasma and urine and 0.2 ml of tissue homogenate could be determined at levels as low as 2, 100 and 20 ng, respectively. The method can be applied to studies on the pharmacokinetics and distribution of pemoline in small animals.

REFERENCES

1 K. Rickels, P.E. Gordon, D.H. Gansman, C.C. Weise, J.A. Pereira-Ogan and P.T. Hesbacher, Clin. Pharmacol. Ther., 11 (1970) 698.
2 Y. Honda and Y. Hishikawa, Curr. Ther. Res., 27 (1980) 429.
3 C.P. Collier, S.J. Soldin, J.M. Swanson, S.M. MacLeod, F. Weinberg and J.G. Rochefort, Clin. Pharmacokin., 10 (1985) 269.
4 F. Sallee, R. Stiller, J. Perel and T. Bates, Clin. Pharmacol. Ther., 37 (1985) 606.
5 C.P. Tomkins, S.J. Soldin, S.M. MacLeod, J.G. Rochefort and J.M. Swanson, Ther. Drug Monit., 2 (1980) 255.
6 L.H. Cummins and J.E. Perry, J. Pharm. Sci., 58 (1969) 762.
7 J.J. Brink and D.G. Stein, Biochem. Pharmacol., 17 (1968) 2365.
8 P.W. Dodge and B. Celarec, Fed. Proc., Fed. Am. Soc. Exp. Biol., 27 (1968) 237.
9 J.C. Libeer and P. Schepens, J. Pharm. Sci., 11 (1978) 419.
10 S.-Y. Chu and L.T. Sennello, J. Chromatogr., 137 (1977) 343.
11 O.J. Igwe and J.W. Blake, J. Chromatogr. Sci., 19 (1981) 617.
12 N.P.E. Vermeulen, D. De Roode and D.D. Breimer, J. Chromatogr., 137 (1977) 333.
13 N.P.E. Vermeulen, M.W.E. Teunissen and D.D. Breimer, J. Chromatogr., 157 (1978) 133.
14 N.P.E. Vermeulen, M.W.E. Teunissen and D.D. Breimer, Br. J. Clin. Pharmacol., 8 (1979) 459.
15 D.J. Hoffman, J. Pharm. Sci., 68 (1979) 445.
16 A.I. Krylov and N.S. Khlebnikova, J. Chromatogr., 252 (1982) 319.
17 O.J. Igwe and J.W. Blake, Drug Metab. Dispos., 11 (1983) 120.

18 W. Gielsdolf, J. Clin. Chem. Clin. Biochem., 20 (1982) 65.
19 G.P. Cartoni and F. Natalizia, J. Chromatogr., 123 (1976) 474.
20 G.P. Cartoni, M. Ciardi, A. Giarrusso and F. Rosati, J. Chromatogr., 202 (1980) 131.
21 K. Nishihara, Y. Kohda, Y. Saitoh, F. Nakagawa and Y. Honda, Ther. Drug Monit., 6 (1984) 232.