ELSEVIER

Journal of Pharmaceutical and Biomedical Analysis
16 (1998) 1029–1035

JOURNAL OF
PHARMACEUTICAL
AND BIOMEDICAL
ANALYSIS

Simplified reversed-phase HPLC method with spectrophotometric detection for the assay of verapamil in rat plasma[1]

C.A. Lau-Cam *, D. Piemontese

St John's University, College of Pharmacy and Allied Health Professions, 8000 Utopia Parkway, Jamaica, New York, 11439, USA

Received 15 November 1996; accepted 31 March 1997

Abstract

A high-performance liquid chromatographic (HPLC) method was developed for the assay of verapamil in rat plasma. After deproteinization of the plasma sample with an acetonitrile-perchloric acid (8:2) mixture containing dextromethorphan, the internal standard, an aliquot of the supernatant was directly analyzed on a cyanopropylsilane column with methanol-acetonitrile-triethylamine acetate buffer (10:30:60) as the mobile phase and detection at 235 mm. At a flow rate of 1.5 ml min^{-1}, a complete analysis was completed in less than 6 min. The method was linear for verapamil concentrations in the range 0.5–10 μg ml^{-1} ($r = 0.9999$). Recoveries for the same drug concentrations from spiked rat plasma ranged from 85.6-93.0% ($n = 8$). The mean RSD values for intraday and interday assay reproducibility ($n = 3$) were, in both cases, less than 0.9%. The limit of detectability was about 0.1 μg ml^{-1}. The method was found useful to monitor the plasma levels of verapamil in rats that had received this drug by the nasal, oral and intravenous routes of administration.

Keywords: Verapamil; Rat plasma; One-step sample preparation; HPLC; Cyanopropylsilane column; Spectrophotometric detection; Pharmacokinetic studies

1. Introduction

Verapamil is a calcium channel blocking agent which has found widespread use in the management of supraventricular tachyarrhythmias, angina pectoris, ischemic heart disease, hypertrophic cardiomyopathy and hypertension [1–3]. Although this compound is well absorbed orally, bioavailability is low (20–35%) because of rapid and extensive hepatic conversion to norverapamil, a biologically active *N*-demethylated metabolite, and a group of at least six major inactive derivatives [2–7]. Hence, alternative routes of administration have been sought [8,9].

This laboratory is currently investigating the intranasal absorption of selected calcium channel blockers in in vivo animal models. Interest in this

* Corresponding author. Tel.: +1 718 9906260.

[1] Presented at the Analysis and Pharmaceutical Quality Section of the Eleventh Annual American Association of Pharmaceutical Scientists Meeting, October 1996, Seattle, Washington, USA.

0731-7085/98/$19.00
PII S0731-7085(97)00117-9

route of administration was elicited by the results of earlier studies showing that the systemic bioavailability of certain calcium antagonists susceptible to a first-pass effect will dramatically improve upon delivery from the nasal cavity [8–11]. Furthermore, absorption studies conducted with animal models are found to be useful to compare drug pharmacodynamics among different routes of administration [8–11], and to predict bioavailability in humans [10].

Several analytical techniques have been suggested for the quantitative determination of verapamil in biological fluids and tissues as part of studies to characterize the pharmacokinetic properties of this drug [4–7,9–41]. For example, verapamil was determined in plasma, serum, urine and tissue homogenates by a reproducible fluorometric method [12]; but this method requires a very large (greater than 4 ml) volume of sample, the sample preparation is lengthy and complicated, and it is interfered by fluorescent inactive metabolites. By the combined used of mass spectrometry with isotopic dilution (mass fragmentography) [13] or of gas chromatography with mass spectrometry [14], plasma verapamil was measured in a sensitive and specific manner, but these techniques require instrumentation which is normally beyond the reach of the average analytical laboratory. Gas liquid chromatography has been successfully applied to the measurement of verapmil and some of its metabolites in biological fluids [15–17]; however this approach makes use of at least 1 ml of blood or plasma sample, and the potential for sample loss is always present since the sample preparation entails repeated back-extractions.

High performance liquid chromatography (HPLC) has been the technique most often applied to the analysis of verapamil in serum or plasma [4–7,9,18–40]. Methods based on the use of a reversed-phase [4–7,9,18–37,40,41], normal phase [38] or ion-exchange [39] column with either spectrophotometric [18–22] or fluorometric [4–7,9,23–41] detection have been described. In the majority of cases, the isolation of verapamil from the biological matrix has consisted of a liquid–liquid extraction from an alkaline sample into a volatile, water-immiscible, organic phase which, after evaporation to dryness is reconstituted in the mobile phase or in a solvent compatible with the mobile phase [7,18–20,24,25,30,32–34,36–39]. Very often, however, the initial extraction is followed by a back-extraction into dilute aqueous acid, which is then injected onto the column [4–6,9,22,23,31]. A more recent alternative to the isolation of verapamil from biological fluids has been liquid–solid extraction [40,41]. When automated, this technique is found more convenient to the slower and less precise liquid–liquid extraction, but the cost involved and need for equipment not readily accessible precludes its wider acceptability.

The purpose of this report is to describe a reversed-phase HPLC method with spectrophotometric detection that will quantify verapamil in a small volume of plasma with no other sample preparation than a deproteinization step. This method was found to represent a simple, rapid, and accurate means of assessing the systemic absorption of verapamil from various sites in a rat model.

2. Experimental

2.1. Apparatus

An isocratic system consisting of a Series 3B liquid chromatograph (Perkin-Elmer, Norwalk, CT), a Spectroflow 783 programmable absorbance detector (Kratos Analytical, Ramsey, NJ), and a LCI-100 laboratory computing integrator (Perkin-Elmer). Samples were introduced automatically with a WISP 710B sample processor (Waters Associates, Milford, MA) or manually through a Model 7125 sample injector fitted with a 100 μl loop (Rheodyne, Cotati, CA).

2.2. Chemicals and solutions

Verapamil hydrochloride and dextromethorphan hydrobromide monohydrate (Sigma, St. Louis, MO) and ($\pm$)-norverapamil hydrochloride (Research Biochemicals International, Natick, MA) were used as received. Acetonitrile, methanol, and water were of HPLC reagent grade

(EM Science, Gibbstown, NJ). Glacial acetic acid, perchloric acid (70%) and triethylamine were of analytical reagent grade (J.T. Baker, Phillipsburg, NJ).

The deproteinizing solution was a mixture of acetonitrile–perchloric acid in a an 8:2 ratio. The internal standard solution was prepared by dissolving dextromethophan hydrobromide in extracting solution to a concentration of about 43 μg ml^{-1}.

2.3. Chromatographic conditions

The mobile phase was a mixture of methanol–acetonitrile–water (10:30:60), to which 0.1 ml of acetic acid and 0.2 ml of triethylamine were added in succession, filtered and degassed prior to use. The flow rate was 1.5 ml min^{-1}. Separations were accomplished at ambient temperature on an analytical Microsorb-MV Cyano column (150 × 4.6 mm i.d., 5 μm particle size, Rainin Instrument Company, Woburn, MA) protected by an Adsorbosil CN guard column (Alltech Associated, Deerfield, IL). A Microsorb-MV C18 (100 × 4.6 mm i.d., 3 μm particle size) column protected by an Adsorbosil C18 guard column was used in studies involving the separation of verapamil from norverapamil. Detection was at 235 nm and 0.05 AUFS.

2.4. Sample extraction

Portions of plasma sample and deproteinizing solution, in a ration of 4:1, were added to a 2 ml polypropylene microcentrifuge tube with a snap-cap. After capping, the tube contents were vortex mixed for 30 s, and the suspension was centrifuged at 4000 rpm for 10 min. A 100 μl portion of the clear supernatant was injected into the liquid chromatograph.

2.5. Method validation

The linearity of the method was investigated by serially diluting a stock aqueous solution of verapamil hydrochloride (1 mg ml^{-1}) with either water or drug-free rat plasma to concentrations of verapamil in the range 2–10 μg ml^{-1}, and subjecting these solutions to the proposed assay method. Samples in plasma were prepared in quintuplicate, while those in water were prepared in triplicate. Calibration curves were constructed by plotting the ratio of the peak areas of verapamil to internal standard against the concentrations of verapamil hydrochloride added.

Analyte recovery was determined by comparing the ratio of the peak areas of verapamil to internal standard for the standard preparations against those of the same preparations in water plus deproteinizing solution. The recovery of the internal standard, dextromethorphan hydrobromide, was studied in similar manner by using serial dilutions of this compound both in blank rat plasma and in water but without adding verapamil.

Intraday reproducibility was evaluated by analysing sets of drug-free rat plasma samples, spiked with concentrations of verapamil hydrochloride in the range 2–10 μg ml^{-1}, at three different time periods. The assessment of interday reproducibility was based on the analysis of the same spiked plasma samples on two consecutive days.

2.6. Animal studies

Sprague-Dawley rats, 400–450 g in weight, were fasted overnight and divided into groups of five animals each. After anesthesia with an intraperitoneal, 50 mg kg^{-1}, dose of pentobarbital sodium, the rats were surgically intervened to expose the left femoral artery, which was cannulated with a piece of PE-50 tubing for blood sampling purposes. A similar cannula was inserted into the femoral vein of rats that were to receive an intravenous dose. An aqueous solution of verapamil hydrochloride, made isotonic with boric acid, was administered orally (PO) by gavage with an oral feeding needle, intransally (IN) using a positive displacement micropipette fitted with a narrow 50 μl tip, or intravenously (IV) through the femoral vein. In all cases, the treatment dose was 3 mg kg^{-1} (PO, IV) or 3 mg kg^{-1} in a 200 μl volume (IN), calculated as verapamil free base. Blood samples were collected from the femoral artery at 5, 15, 30, 60, 120 and 180 min

after drug administration into heparinized polypropylene test tubes, and subjected to immediate centrifugation at 5000 rpm for 10 min. The clear plasma layers were transferred to clean test tubes, using disposable Pasteur pipettes, and then analyzed for their verapamil contents. The peak area ratio for an unknown sample was converted to concentration by reference to a calibration curve of verapamil constructed with drug-free pooled rat plasma.

3. Results and discussion

In the present study plasma deproteinization was accomplished by the addition of an (8:2) acetonitrile–perchloric acid mixture. In comparison to procedures in which protein removal is effected with a neat organic solvent (i.e. acetonitrile [28,35], methanol [26], 2-methoxy-2-methylpropane [25]) or a mixture of organic solvent (i.e., isopropyl ether–butanol [18]), the one suggested here offers a number of advantages. For example, since maximal protein precipitation with any of these treatments is attained at precipitant to plasma ratios ≥ 1.0 [42], their addition to a biological sample will result in a significant dilution of the sample and, thereby, in a decrease in the limit of sensitivity of the method. For this reason, the detection of verapamil and its metabolites in HPLC methods utilizing those approaches is fluorometric [26–28]. On the other hand, it was verified that the use of an acetonitrile-perchloric acid mixture yielded protein-free plasma sample at volumes much smaller than those of acetonitrile or methanol. Thus, in a typical experiment, only about 25 μl of the mixture was sufficient to satisfactorily deproteinize a 100 μl sample of plasma while keeping the dilution of the analyte within the limit of spectrophotometric detectability. This approach is simpler than the procedure in which protein precipitation by acetonitrile was enhanced by the subsequent addition of a weighed quality of an inorganic sulfate [19]. No evidence of significant loss in resolution or in column efficiency was noted throughout the study.

Published mobile phases for the reversed-phase HPLC analysis of verapamil have contained a corrosive salt [25], a cumbersome to prepare buffer [28], or an ion-pair reagent [4,6,22,32,38,40,41]. The mobile phase suggested here does not require any of these components and can be readily prepared by simple admixture. The addition of an organic base such as triethylamine was found to improve peak shape [29]. Representative chromatograms of a blank rat plasma sample and of a rat plasma sample containing verapamil and the internal standard, obtained with the mobile phase flowing at the rate of 1 ml min^{-1}, are shown in Fig. 1(A and B), respectively. Under these conditions, approximate elution times for dextromethorphan and verapamil were about 5.2 and 7.5 min, respectively ($R = 3.54$). When analysing multiple samples, use of a faster flow rate will shorten the analysis time without appreciable loss in resolution. For example, at 1.5 ml min^{-1}, verapamil eluted at about 4.65 min and the internal standard at about 2.9 min, as seen in Fig. 1B, inset. To determine if the proposed method will be able to resolve a mixture of verapamil with its physiologically active metabolite, norverapamil, a rat drug-free plasma sample was spiked with the two compounds and put through the deproteinization procedure. As seen in Fig. 1C, norverapamil (7.07 min) eluted ahead of verapamil (7.53 min) as a partially resolved peak (R from verapamil $= 0.71$, R from dextromethorphan $= 3.05$). In the event that the resolution of a mixture of the two compounds is deemed necessary, this objective can be easily realized by substituting the cyanopropylsilane column with a more retentive one, such as a C18, while maintaining all of the other suggested experimental conditions. A separation attained on this column with the mobile phase flowing at the rate of 1 ml min^{-1} is depicted in Fig. 1C, inset (R between norverapamil and verapamil $= 1.53$; R between internal standard and verapamil $= 4.50$; R between internal standard and norverapamil $= 3.36$).

Standard curves for verapamil were linear over the range 0.5–10 μg ml^{-1} both in water and in plasma. The regression analysis line equations (correlation coefficients) in water (triplicate samples) and rat plasma (quintuplicate samples) were $y = 0.8815x + 0.0410$ $(r = 0.9992)$ and $y =$

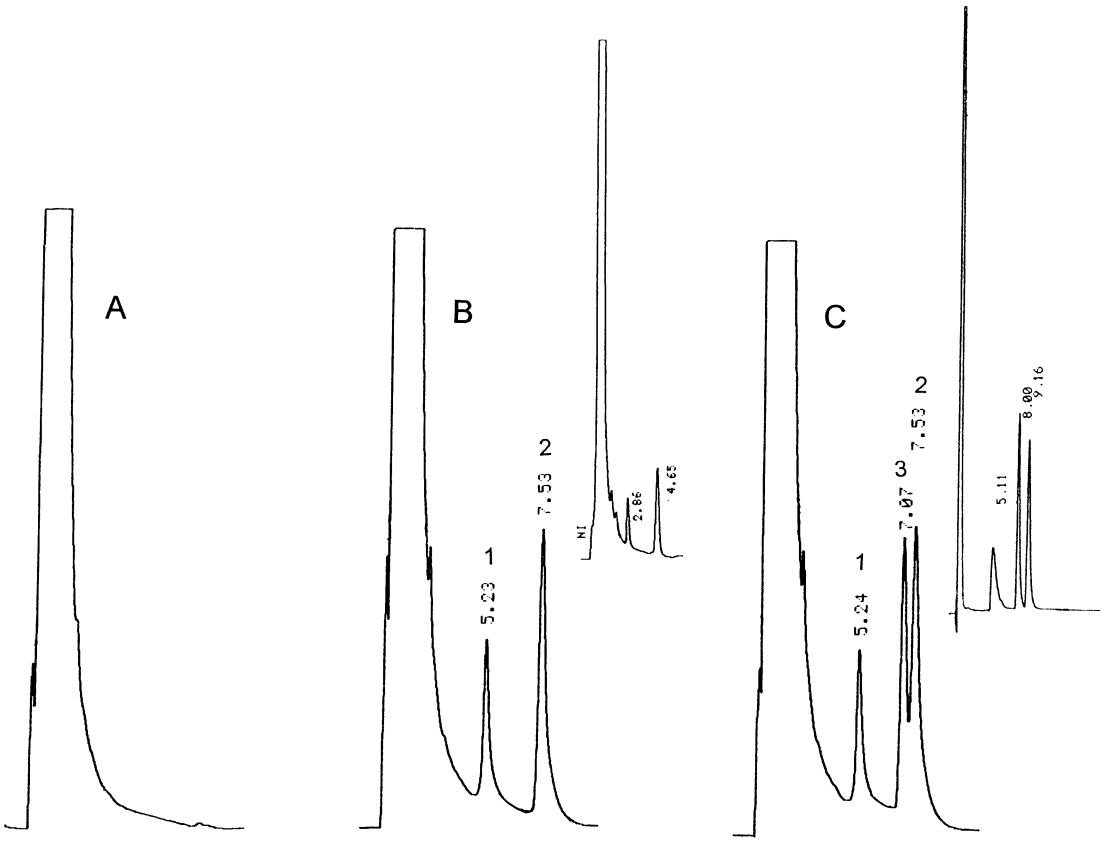

Fig. 1. Liquid chromarograms of (A) blank rat plasma, (B) rat plasma containing verapamil and the internal standard, and (C) rat plasma spiked with verapamil, norverapamil and the internal standard. All chromatograms were obtained on a cyanopropylsilane column except for that in the inset of 1C, which was derived using a C18 column. In all cases, the flow rate was 1 ml min^{-1}, except in the case of the inset of 1B, which was obtained at 1.5 ml min^{-1}. Key: 1, dextromethorphan, the internal standard; 2, norverapamil; 3, verapamil.

$0.7030x + 0.0740$ ($r = 0.9999$), respectively. In both cases the intercepts were not significantly different from zero. The minimum quantifiable level of verapamil in rat plasma, at a signal to noise ratio of 2:1, was 0.25 μg ml^{-1} when using 100 μl of plasma. The minimum amount of analyte producing a discernible peak in the chromatogram was about 0.1 μg ml^{-1}.

The accuracy of the proposed method was established by comparing the peak area ratios for levels of verapamil added to drug-free rat plasma between 2–10 μg ml^{-1} and the peak area ratio for the same drug levels in water plus extracting medium. As summarized in Table 1, recoveries were in general better than 85% (range 85.6–93.0%, $n = 3$) over the concentration range 12.5–100 μg ml^{-1}.

The intraday reproducibility of the proposed assay method was evaluated by comparing the peak area ratios obtained a three different time periods for three sets of plasma samples serially spiked with verapamil at the 2–10 μg ml^{-1} levels. The results of this study are summarize in Table 2. The mean RSD value was 0.86% (range 0.90–1.20%). To assess interday reproducibility, the same sets of spiked plasma were assayed on 2

Table 1
Recovery of verapamil from spiked rat plasma

Added (μg ml^{-1})	n	Found mean ± S.D. (μg ml^{-1})	Recovery mean ± S.D. (%)	RSD (%)
2	8	1.71 ± 0.01	85.6 ± 0.58	0.68
4	8	3.72 ± 0.03	93.0 ± 0.73	0.78
6	8	5.40 ± 0.04	90.0 ± 0.69	0.77
8	8	7.30 ± 0.06	90.9 ± 0.78	0.86
10	8	9.04 ± 0.10	90.4 ± 1.04	1.15

Table 2
Intraday and interday precision data of proposed HPLC method

Concentration (μg ml^{-1})	n	Intraday area ratio		Interday area ratio	
		Mean ± S.D.	RSD (%)	Mean ± S.D.	RSD (%)
2	3	4.44 ± 0.04	0.90	4.45 ± 0.03	0.75
4	3	8.44 ± 0.05	0.55	8.47 ± 0.07	0.81
8	3	16.11 ± 0.12	0.77	16.11 ± 0.14	0.84
10	3	21.18 ± 0.25	1.20	21.31 ± 0.21	1.00

consecutive days, with the results reported in the same table. The mean RSD value was 0.85% (range 0.75–1.00%).

The applicability of the method to systemic absorption studies from different routes of administration was investigated by dosing rats with equidoses of verapamil by the PO, IN and IV routes, and monitoring the ensuing drug plasma levels over a period of 6 h. Fig. 2 shows the temporal changes in plasma drug levels obtained by the proposed HPLC method. From these time profiles, it is evident that IN dosing of verapamil will result in a greater systemic bioavailability than by the PO route. The details of the pharmacokinetic data will be described in a separate publication. Neither norverapamil nor interfering rat plasma constituents were observed throughout the study.

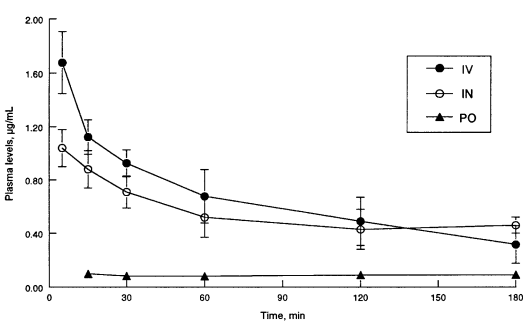

Fig. 2. Mean plasma concentrations of verapamil in rats following intravenous (IV), intranasal (IN) and oral (PO) administrations of a dose of verapamil hydrochloride equivalent to 3 mg kg^{-1} of the free base. Vertical lines represent the S.D. values for $n = 5$.

4. Conclusions

The HPLC method with spectrophotometric detection described here analyzes verapamil in rat plasma in less than 200 µl of sample, and in as little as 6 min, with reasonable sensitivity, and good accuracy and precision. The sample preparation consists of a simple one-step deproteinization, the internal standard is readily available, and the mobile phase can be prepared by the simple admixing of its components. This method should be useful in pharmacokinetic studies involving small animal models and numerous samples.

References

[1] S.R. Hamann, G.D. Todd, R.G. McAllister Jr., Pharmacology 27 (1983) 1–8.
[2] S.R. Hamann, R.A. Blouin, R.G. McAllister Jr., Clin. Pharmacokin. 29 (1984) 264–268.
[3] Physicians' Desk Reference, 48th ed., Medical Economics Data Production Co., Montvale, NJ, 1984, pp. 1132–1137.
[4] S.R. Harapat, R.E. Kates, J. Chromatogr. 170 (1979) 385–390.
[5] P.A. Kapur, T. Law, E. Watson, J. Chromatogr. 337 (1985) 160–165.
[6] M. Kuwada, T. Tateyama, J. Tsutsumi, J. Chromatogr. 223 (1981) 507–511.
[7] D.L. Bremseth, J.J. Lima, J.J. Mac Kichan, J. Liq. Chromatogr. 11 (1988) 2731–2749.
[8] Y. Yazan, A.Y. Özer, K. Erol, Drug Dev. Ind. Pharm. 22 (1996) 281–284.
[9] T.H. Arnold, R.L. Tackett, J.J. Vallner, Biopharm. Drug Disp. 6 (1985) 447–454.
[10] G.C. Visor, E. Bajka, E. Benjamin, J. Pharm. Sci. 75 (1986) 44–46.
[11] G.C. Visor, B. Schuessler, J. Thompson, T. Ling, Drug Dev. Ind. Pharm. 13 (1987) 1329–1343.
[12] R.G. McAllister, S.M. Howell, J. Pharm. Sci. 65 (1979) 431–432.
[13] B. Spiegelhalder, M. Eichelbaum, Arzneim.-Forsch./Drug Res. 27 (1977) 94–97.
[14] P.A. Hynning, P. Anderson, U. Bondesson, L.O. Boréus, Clin. Chem. 34 (1988) 2502–2503.
[15] B.G. Woodcock, P. Wörner, N. Rietbrock, L. Schwabe, K.H. Frömming, Arzneim.-Forsch./Drug Res. 32 (1982) 1567–1571.
[16] H.G. Hege, Arzneim.-Forsch./Drug Res. 29 (1979) 1681–1684.
[17] U.A. Shukla, P.L. Stetson, W.D. Ensminger, J. Chromatogr. 342 (1985) 406–410.
[18] G. Mazzi, Chromatographia 24 (1987) 313–316.
[19] A.M. Rustum, J. Chromatogr. 528 (1990) 480–486.
[20] H. Fieger, G. Blaschke, J. Chromatogr. 575 (1992) 255–260.
[21] Z. Zhu, G. Hu, Zhongguo Yaoxue Zazhi 27 (1992) 280–282; Anal. Abstr. 55 (1993) 10G262.
[22] D.R. Rutledge, A.H. Abadi, L.M. Lopez, J. Chromatogr. Sci. 32 (1994) 153–156.
[23] T.M. Jaouni, M.B. Leon, D.R. Rosing, H.M. Fales, J. Chromatogr. 182 (1980) 473–477.
[24] E. Watson, P.A. Kapur, J. Pharm. Sci. 70 (1981) 800–801.
[25] S.C.J. Cole, R.J. Flanagan, A. Johnson, D.W. Holt, J. Chromatogr. 218 (1981) 621–629.
[26] C.K. Lim, J.M. Rideout, J.W.S. Sheldon, J. Liq. Chromatogr. 6 (1983) 887–893.
[27] A.T. Kacprowicz, R.O. Fullinfaw, R.W. Bury, J. Chromatogr. 337 (1985) 412–415.
[28] E.M. Niazy, H.W. Jun, Anal. Lett. 18 (1985) 1103–1112.
[29] S.A. Hamadi, S.-L. Chang, P.J. Wedlund, Anal. Lett. 19 (1986) 1297–1310.
[30] S.M. Johnson, S.K.W. Khalil, J. Liq. Chromatogr. 10 (1987) 1187–1201.
[31] C.M.M. Haring, A.A.T.M.M. Vinks, Ziekenhuisfarmacie 2 (1986) 86–88; Anal. Abstr. 49 (1987) 4D82.
[32] C. Horne, H. Spahn, E. Mutschler, H. Knauf, Arzneim. Forsch./Drug Res. 37 (1987) 956–959.
[33] X. Dai, S. Li, Sepu 5 (1987) 129–130; Anal. Abstr. 50 (1988) 1D95.
[34] Z.B. Salama, C. Dilger, W. Czogalla, R. Otto, H. Jaeger, Arzneim.-Forsch./Drug Res. 39 (1989) 210–215.
[35] C. Köppel, A. Wagemann, J. Chromatogr. 570 (1991) 229–234.
[36] A.K. Rasymas, H. Boudoulas, J. Mac Kichan, J. Liq. Chromatogr. 15 (1992) 3013–3029.
[37] B.L. Carter, M.A. Noyes, R.W. Demmler, Pharmacotherapy 13 (1993) 359–368.
[38] J.A. Pieper, D.R. Rutledge, J. Chromatogr. Sci. 26 (1988) 473–477.
[39] V.K. Piotrovskii, D.O. Rumiantsev, V.I. Metelitsa, J. Chromatogr. 275 (1983) 195–200.
[40] Ph. Hubert, P. Chiap, A. Ceccato, I. Bechet, R. Sibenaler-Dechamps, P. Maes, J. Crommen, J. Pharm. Biomed. Anal. 10 (1992) 937–942.
[41] Ph. Hubert, J. Crommen, J. Liq. Chromatogr. 17 (1994) 2147–2170.
[42] J. Blanchard, J. Chromatogr. 226 (1981) 455–460.

.