Available online at www.sciencedirect.com
SCIENCE DIRECT®
Journal of Chromatography A, 1031 (2004) 289–294
JOURNAL OF CHROMATOGRAPHY A
www.elsevier.com/locate/chroma

High-performance liquid–chromatographic determination of rifampicin in plasma and tissues

Ignacio Calleja, María J. Blanco-Príeto, Noelia Ruz, María Jesús Renedo, María Carmen Dios-Viéitez*

Departamento de Farmacia y Tecnología Farmacéutica, Facultad de Farmacia, Universidad de Navarra, C/Irunlarrea 1, 31080 Pamplona, Spain

Abstract

A HPLC–UV method has been developed for assaying rifampicin in plasma and liver. The assay involved a liquid–liquid extraction procedure with dichloromethane–pentane (1:1). An Ultrabase-C_{18} column and a simple mobile phase consisting of a water (pH 2.27)–acetonitrile (40:60, v/v) mixture were used. The flow-rate was 1 ml/min and the effluent was monitored at 333 nm. Results from the HPLC analyses showed that the assay method is linear in the ranges 0.1–1 and 1–50 μg/ml for plasma, and 0.6–40 μg/g for liver. Intra- and inter-day R.S.D. were below 15% for all the sample types. Recoveries averaged 83 and 95% for plasma and liver, respectively. The method is being successfully applied to determine rifampicin in plasma and liver samples taken during pharmacokinetic studies in rats.

Keywords: Rifampicin; Antibiotics

1. Introduction

Rifampicin (Fig. 1), a complex semisynthetic macrocyclic antibiotic derived from *Streptomyces mediterranei*, is a member of the rifamycin class of antibiotics [1] used for the treatment of tuberculosis and other infectious diseases [2–5]. It is categorised one of the first line antituberculous agents, however various side effects such as hepatotoxicity, allergic rashes, lack of appetite, nausea or immunological disturbances have been reported associated with the administration of the drug [5–7]. Tuberculosis remains a major health public problem and is the single most deadly infectious disease. It kills approximately two million people each year and therefore new formulations of rifampicin are being studied with the aim of improving the therapeutic index of the drug by altering its plasma and tissue distribution profiles [7–9].

In this sense, recently different rifampicin delivery systems (microparticles, nanoparticles) have been developed by our group. In order to characterise the pharmacokinetics of rifampicin after administration of these new formulations during preclinical studies, the drug levels in plasma as well as tissue samples need to be determined. It was therefore necessary to develop a sensitive, accurate and reproducible analytical method to analyse these samples.

Numerous methods have been previously published for the analysis of rifampicin in biological fluids (plasma, serum, urine, etc.). The most common described procedures to determine rifampicin in plasma include microbiological assays [10], HPTLC [11], HPLC thermospray mass spectrometry and electrospray mass spectrometry [12] and some other HPLC methods. However, most of them needed relatively large volumes of sample (≥0.5 ml) and/or lack of sensitivity [13–23], consequently their application in pharmacokinetic studies in small laboratory animals is unsatisfactory. In addition, some of these methods involve complex or very long extraction procedures, which notably increases the analysis time [13,15].

No chromatographic methods have to date been published for the quantitation of liver concentrations of rifampicin, which are very important to estimate in rifampicin delivery systems in order to determine the distribution of the drug in this tissue. Only microbiological assay procedures [10] have been described.

In this paper an accurate, reproducible and sensitive HPLC method is described for the assay of rifampicin in plasma and liver samples. The assay involves a liquid–liquid extraction, the application of an Ultrabase-C_{18} reversed-phase column

* Corresponding author. Tel.: +34-948-425600x6442; fax: +34-948-425649.
E-mail address: mcdios@unav.es (M.C. Dios-Viéitez).

0021-9673/$ – see front matter © 2004 Elsevier B.V. All rights reserved.
doi:10.1016/j.chroma.2003.12.041

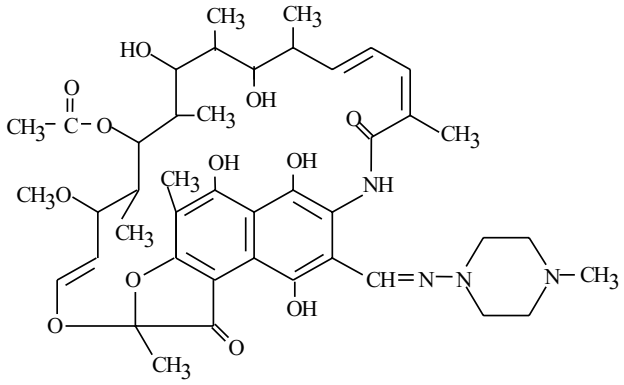

Fig. 1. Chemical structure of rifampicin.

for the chromatographic separation and the use of UV detection. Furthermore, to prevent oxidation of rifampicin during the process, butylhydroxytoluene (0.02%) was added to the solvent used for preparing the drug standards solutions as well as to the extraction solvent. The developed HPLC assay method was applied to quantitate rifampicin in plasma and liver samples obtained during preliminary pharmacokinetic studies in rats.

2. Experimental

2.1. Chemicals and biological matrixes

Rifampicin (RIF) was purchased from Sigma–Aldrich (St. Louis, MO, USA) and the purity was at least 95%. Acetonitrile (ACN) and dichloromethane (both HPLC grade) were obtained from Merck (Darmstadt, Germany). *n*-Pentane (99% spectroscopy grade, Spectrosol), orthophosphoric acid and disodium hydrogenphosphate anhydrous were provided by Scharlau (Barcelona, Spain). Buthylhydroxytoluene (BHT) and citric acid anhydrous were purchased from Panreac Química (Barcelona, Spain). Ascorbic acid (sodium salt), was supplied by Sigma–Aldrich. Deionized water was procured from a Milli-RX-45 water purification system (Millipore, Bedfort, MA, USA).

A pH 4.5 sodium phosphate buffer (100 ml) was prepared by mixing 56.4 ml of citric acid 0.1 M and 43.6 ml of disodium hydrogenphosphate 0.2 M.

Drug-free rat plasma and liver were obtained from healthy male Wistar rats (weight, 230–260 g) provided from Laboratory Animals Service of CIFA (Universidad de Navarra, Pamplona, Spain).

2.2. Standard solutions

Stock standard solutions of RIF (1 and 1.2 mg/ml) were prepared by dissolving 10 and 12 mg of the drug in 10 ml of ACN (containing 0.02% BHT), respectively. Working standard solutions of RIF (concentrations ranging from 0.2 to 100 μg/ml) were obtained by diluting the stock solutions with ACN (0.02% BHT). The RIF standard solutions were stored protected from light at 4 °C, and under these conditions the drug was found to be stable for at least 3 weeks.

Calibration standards for plasma and liver homogenate (for liver homogenate preparation see Section 2.4.2) were prepared by adding different volumes of RIF working standard solutions to drug-free plasma and liver homogenate. Plasma and liver homogenate calibration standards for RIF were prepared at concentrations of 0.05, 0.1, 0.25, 0.5, 0.75, 1, 5, 10, 20, 25, 30, 40 and 50 μg/ml, and 0.25, 0.3, 0.6, 1.25, 2.5, 5, 10, 20 and 40 μg/g of tissue, respectively. Calibration standards for biological samples were prepared on the required day.

2.3. Chromatographic systems

The HPLC analyses were performed using an Agilent 1100 system (Waldbronn, Germany) equipped with a quaternary pump, an autosampler, a heated column compartment and a diode-array detector. System management and data acquisition were performed by the HP ChemStation 3D software used with a HP personal computer.

Separation was achieved with an Ultrabase-C_{18} reversed-phase column (250 mm × 4.6 mm i.d., 5 μm particle size) (Scharlau, Barcelona, Spain), preceded by an ODS guard column (Teknokroma, Barcelona, Spain). The mobile phase consisted of water (pH 2.27 adjusted with orthophosphoric acid)–acetonitrile (40:60, v/v) at a flow-rate of 1 ml/min. The chromatography was carried out at 25 °C and the eluate was monitored at 333 nm.

2.4. Sample preparation procedures

2.4.1. Plasma

To a 100 μl rat plasma sample (ice cooled) 50 μl of ACN (BHT 0.02%) were added. After vortex mixing (30 s) 3 ml of dichloromethane–*n*-pentane (1:1) (BHT 0.02%) were used for extraction of RIF by vortexing for 60 s. The mixture was centrifuged at 2604 × *g* for 5 min (4 °C), the organic layer transferred to a glass conical tube and evaporated to dryness in a vortex evaporator (Buchler Instruments, Fairfield, NJ, USA). The extraction procedure was repeated and the total residue obtained was reconstituted in 100 or 200 μl of ACN (BHT 0.02%) according to the expected concentration of RIF: a volume of 100 μl was used for concentrations of 1 μg/ml or lower, and a volume of 200 μl for concentrations higher than 1 μg/ml. Aliquots of these solutions (75 and 100 μl, respectively) were injected into the chromatograph after Millipore Millex-HV filtration (0.45 μm).

2.4.2. Liver

To a 0.5 g rat liver sample 2 ml of sodium phosphate buffer (pH = 4.5) containing 10^{-3} M sodium ascorbate were added. The sample was homogenized by using an ultraturrax-T25 dispersing apparatus (24,000 rpm, 3 min)

(Janke & Kunkel, Staufen, Germany). An aliquot of the homogenate (300 μl) was transferred to a glass conical tube, placed on ice, and 50 μl of ACN (BHT 0.02%) were added and vortex mixed (30 s). The mixture was extracted with 3 ml of dichloromethane–*n*-pentane (1:1) (BHT 0.02%) by vortexing for 60 s. After centrifugation at 2604 × *g* for 5 min (4 °C), the supernatant was transferred to a glass conical tube and evaporated to dryness in a vortex evaporator. The extraction procedure was repeated and the total residue was redissolved in 200 μl of ACN (BHT 0.02%). After filtration, an aliquot of this solution (50 μl) was injected into the HPLC system.

2.5. Recovery

The recoveries of RIF from plasma and liver homogenate were determined by spiking an equal amount of the drug into the corresponding blank sample and ACN (BHT 0.02%) (solvent used in the final chromatographic step). Recoveries at three concentration levels for plasma and liver homogenate were studied in six-fold for each sample type.

Percentage recoveries were calculated by comparing the absolute responses (peak areas) of RIF from sample extracts to the absolute responses (peak areas) of non-extracted standards [RIF in ACN (BHT 0.02%) for plasma and liver].

2.6. Linearity, precision and accuracy

Calibration curves of RIF in plasma and liver homogenate were established over the following concentration ranges: 0.1–1 and 1–50 μg/ml for plasma, and 0.6–40 μg/g of tissue for liver homogenate. For construction of the calibration curves in plasma, eight different calibration standards were prepared and processed for the range 1–50 μg/ml, and five for the range 0.1–1 μg/ml. For construction of the calibration curve in liver homogenate, seven different calibration standards were prepared and processed. All calibration curves were done in triplicate. Peak areas of RIF versus the corresponding drug concentrations were plotted.

Precision (intra- and inter-day variation) was evaluated by analyzing six replicate plasma and liver homogenate samples at the following concentrations: 1, 10 and 50 μg/ml for plasma, and 1.25, 5 and 20 μg/g of tissue for liver homogenate. The variability was expressed as the relative standard deviation (R.S.D.). To be acceptable, the values should be less than 15% at all concentrations [24].

Accuracy of the assay method was calculated from the same samples as those used for intra- and inter-day variation studies. The accuracy was expressed as %bias. To be acceptable, the values should be within ±15% at all concentrations [24].

$$\%\text{Bias} = \frac{(\text{observed concentration} - \text{nominal concentration})}{\text{nominal concentration}} \times 100$$

2.7. Detection and quantitation limits

Limits of detection (LOD) and quantitation (LOQ) were estimated from the signal-to-noise ratios [25]. The detection limit was defined as the lowest concentration level resulting in a peak area of three times the baseline noise. The quantitation limit was defined as the lowest concentration level that provided a peak area with a signal-to-noise ratio higher than ten, with a precision less than 15% (R.S.D.) and an accuracy within ±15% (bias).

3. Results and discussion

3.1. Method development and validation

The Ultrabase-C_{18} column and the simple mobile phase used were found to be appropriate for the analysis of RIF. The retention time for RIF was around 4 min under the assay conditions described. Peak symmetry was good and band spreading minimal.

Different ratios and flow-rates of the mobile phase as well as water pHs and column temperatures were studied in order to shorten the retention time of RIF and to improve peak symmetry. However, the optimal assay conditions were found when using a ratio of 40:60 (v/v) for water–ACN, the pH of water adjusted to 2.27, the column temperature set at 25 °C and the flow-rate 1 ml/min. The use of 333 nm wavelength improved the resolution of the RIF without any interference from endogenous plasma and liver compounds.

Fig. 2 illustrates the chromatograms obtained from blank rat plasma and liver extracts and plasma and liver specimens spiked with RIF. No interfering peaks were observed in drug-free plasma and tissue samples (Fig. 2).

Recovery of RIF averaged 83% from plasma at concentrations of 0.1–50 μg/ml and 95% from liver homogenate at concentrations of 0.6–40 μg/g of tissue. The results are summarized in Table 1.

The peak areas of RIF showed a linear relationship to drug concentrations within the ranges 0.1–1 and 1–50 μg/ml for plasma, and 0.6–40 μg/g of tissue for liver homogenate. The parameters of the calibration curves in plasma were: $y = 241.82x - 0.50$ and $y = 232.35x - 9.93$ for the calibration ranges 0.1–1 and 1–50 μg/ml, respectively (x is the concentration of RIF; y is the peak area). The parameters of the calibration curve in liver homogenate were $y = 20.26x - 5.74$. Standard deviations of the slopes for the calibration curves in plasma were 17.28 and 2.54 for the calibration ranges 0.1–1 and 1–50 μg/ml, respectively, and the standard deviations of the corresponding intercepts were 0.33 and 4.79. Standard deviations of the slope and the intercept for the calibration curve in liver homogenate were 0.34 and 4.80, respectively. The results of linear regression analysis showed that the correlation coefficients of all calibration curves were ≥0.995.

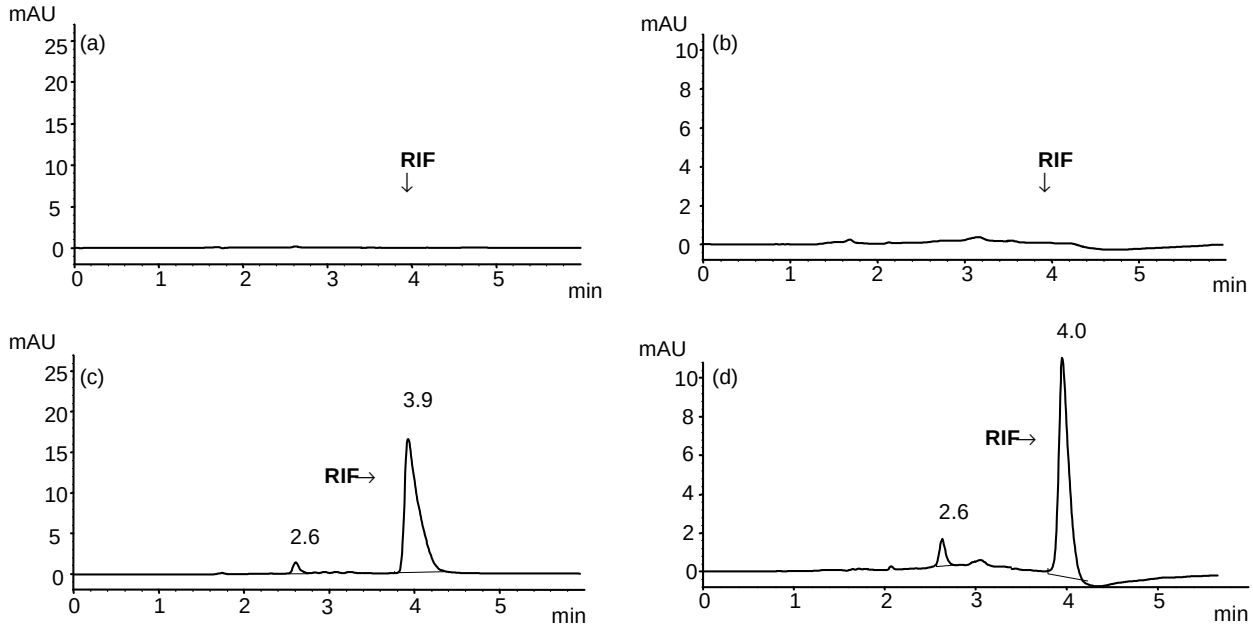

Fig. 2. Typical chromatograms of drug-free plasma (a) and liver (b) extracts. Chromatograms of plasma (c) and liver (d) samples spiked with rifampicin (10 μg/ml and 5 μg/g, respectively).

Table 1
Recoveries of rifampicin from plasma and liver

Plasma		Liver	
RIF (μg/ml)	Recovery (%), mean ± S.D. ($n = 6$)	RIF (μg/g)	Recovery (%), mean ± S.D. ($n = 6$)
50	83.89 ± 2.05	40	93.01 ± 2.45
1	82.84 ± 1.26	5	100.60 ± 0.50
0.1	81.41 ± 5.40	0.6	90.76 ± 7.23

The intra-day variabilities of the assay method for plasma and liver are shown in Table 2 and the corresponding inter-day variabilities in Table 3. The data indicate that the assay method is reproducible within the same day and within different days; R.S.D. are less than 15% for both sample types over the concentration ranges assayed.

The values of accuracy of the assay method in determining RIF concentrations in spiked plasma and liver homogenate specimens are shown in Tables 2 and 3. The deviation from theoretical values is below 15% at all concentrations levels for each sample type.

The detection limits of the assay method were found at 0.025 μg/ml for plasma and 0.06 μg/g for liver, and the estimated limits of quatitation were 0.05 μg/ml and 0.25 μg/g, respectively. These quantitation limits were confirmed, in separate experiments, using calibrators with nominal concentrations of 0.05 μg/ml (plasma) and 0.25 μg/g (liver), obtaining coefficients of variation <15% (Table 2).

3.2. Application of the method to pharmacockinetic and tissue distribution studies in rats

The described HPLC method is sensitive enough for the quantitative determination of RIF in plasma and liver samples obtained during preclinical studies. The small amount of plasma required makes this procedure highly suitable for pharmacockinetic studies in small laboratory animals.

As an example, the developed method has been applied in a preliminar pharmacokinetic study to determine plasma and liver concentrations of RIF following a single intravenous bolus administration (20 mg/kg) of the drug (Rifaldin I.V.®, Hoechst Marion Merrell S.A., Barcelona, Spain) in rats

Table 2
Intra-day precision and accuracy of the HPLC assay for rifampicin

	Plasma				Liver			
	50 (μg/ml)	10 (μg/ml)	1 (μg/ml)	0.05 (μg/ml)[a]	20 (μg/g)	5 (μg/g)	1.25 (μg/g)	0.25 (μg/g)[a]
Mean ± S.D. ($n = 6$)	51.9 ± 1.30	9.6 ± 0.10	1.1 ± 0.064	0.051 ± 0.0063	20.8 ± 0.540	4.9 ± 0.020	1.23 ± 0.080	0.259 ± 0.0169
R.S.D. (%)	2.50	1.04	5.82	12.35	2.60	0.41	6.50	6.52
Bias (%)	3.8	−4.0	10.0	2.0	4.0	−2.0	−1.6	3.6

[a] Limits of quatitation (LOQs).

Table 3
Inter-day precision and accuracy of the HPLC assay for rifampicin

	Plasma			Liver		
	50 (μg/ml)	10 (μg/ml)	1 (μg/ml)	20 (μg/g)	5 (μg/g)	1.25 (μg/g)
Mean ± S.D. ($n = 6$)	53.3 ± 3.10	9.9 ± 0.35	1.14 ± 0.070	21.2 ± 0.13	4.9 ± 0.10	1.33 ± 0.05
R.S.D. (%)	5.82	3.53	6.14	0.61	2.04	3.76
Bias (%)	6.6	−1.0	14.0	6.0	−0.02	6.4

(male Wistar rats weighing 230–260 g). The animals were anaesthetized with ether and sacrificed by cervical dislocation. Arterial blood (±5 ml) was withdrawn from abdominal aorta at the following times after drug injection: 5, 10, 15 and 30 min and 1, 3, 6, 12, 24 and 48 h (three animals were used for each time point). The plasma was separated by centrifugation (2604 × g for 10 min at 4 °C) and L-ascorbic acid (0.1 mg/ml) was added to prevent RIF oxidation [26]. At the time of killing, the liver was removed, dried and weighed. All biological specimens were frozen at −20 °C until the time of analysis; under these conditions the drug was found to be stable for at least 2 weeks. For determination of the RIF plasma and liver concentrations, the samples were thawed at room temperature and prepared according to the procedures described above. Plasma samples corresponding to 12, 24 and 48 h were prepared and processed likewise the calibration standards corresponding to concentration of 1 μg/ml or lower (calibration curve over the range 0.1–1 μg/ml was used to quantitate RIF in those plasma samples). Typical chromatograms obtained from post-dose plasma and liver extracts are shown in Fig. 3. The method is being used to determine plasma and liver concentration-time profiles of RIF following single intravenous bolus administration of drug delivery systems loaded with this antibiotic. All these results will be the subject of further publications.

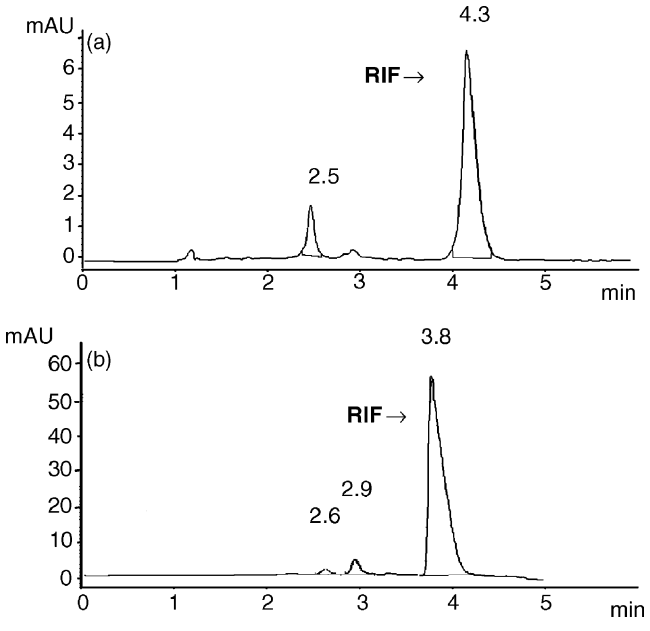

Fig. 3. Chromatograms of post-dose plasma (a) and liver (b) specimens obtained at 10 and 60 min, respectively, after intravenous bolus administration of rifampicin (Rifaldin I.V.®, 20 mg/kg) in rat. The concentration found in plasma was 4.9 μg/ml and in liver 52 μg/g.

In conclusion, the rapidity, simplicity and efficacy of the developed method as well as the short retention time of RIF permit the analysis of a large number of plasma samples in a short time period providing a fast and inexpensive method for therapeutic monitoring in clinical laboratories.

Acknowledgements

The authors wish to thank the Basque Government (Departamento de Educación, Universidades e Investigación) for the fellowship grant.

References

[1] N. Maggi, C.R. Pasqualucci, R. Ballota, P. Sensi, Chemotherapia 11 (1966) 285.
[2] R.J.W. Rees, J.M.H. Pearson, M.F.R. Waters, Br. Med. J. 1 (1970) 89.
[3] G. Binda, E. Domenichini, A. Gottari, Arzneim.-Forsch/Drug Res. 17 (1971) 1907.
[4] R. Pähkla, J. Lambert, P. Ansko, P. Winstanley, P.D.O. Davies, R.-A. Kiivet, J. Clin. Pharm. Ther. 24 (1999) 219.
[5] N. Tsankov, I. Angelova, Clin. Dermatol. 21 (2003) 50.
[6] P. Deol, G.K. Khuller, Biochim. Biophys. Acta 1334 (1997) 161.
[7] M. Gallieni, P. Braidotti, M. Cozzolino, S. Romagnoli, P. Carpani, Int. J. Artificial Organs 22 (1999) 477.
[8] W. Zhang, X. Jiang, J. Hu, C. Fu, J. Microencapsulation 17 (2000) 785.
[9] P. O'Hara, A.J. Hickey, Pharm. Res. 17 (2000) 955.
[10] T. Bruzzese, C. Rimaroli, A. Bonabello, G. Mozzi, S. Ajay, N.D. Cooverj, Arzneim.-Forsch/Drug Res. 50 (2000) 60.
[11] A.P. Argekar, S.S. Kunjir, K.S. Purandare, J. Pharm. Biomed. Anal. 14 (1996) 1645.
[12] W.A. Korfmacher, J. Bloom, M.I. Churchwell, T.A. Getek, E.B. Hansen, C.L. Holder, J. Chromatogr. Sci. 31 (1993) 498.
[13] B. Ratti, R.R. Parenti, A. Toselli, L.F. Zerilli, J. Chromatogr. 225 (1981) 526.
[14] K. Chan, Methods Find. Exp. Clin. Pharmacol. 8 (1986) 721.
[15] J. Woo, C.L. Wong, R. Teoh, K. Chan, J. Chromatogr. 420 (1987) 73.
[16] K. Chan, J. Liq. Chromatogr. 11 (1988) 1051.
[17] M. Ishii, H. Ogata, J. Chromatogr. 426 (1988) 412.
[18] A.B.M. Jamaluddin, G. Sarwar, M.A. Rahim, M.K. Rahman, J. Chromatogr. 525 (1990) 495.
[19] A. El Yazigi, D.A. Raines, Pharm. Res. 9 (1992) 812.
[20] K.J. Swart, M. Papgis, J. Chromatogr. 593 (1992) 21.

[21] A. Walubo, P. Smith, P.I. Folb, J. Chromatogr. B 658 (1994) 391.
[22] S.A. Benetton, E.R.M. Kedor-Hackman, M.I.R.M. Santoro, V.M. Borges, J. Liq. Chromatogr. Rel. Technol. 21 (1998) 3215.
[23] J.E. Conte, E. Lin, E. Zurlinden, J. Chromatogr. Sci. 38 (2000) 72.
[24] F. Bressolle, M. Bromet-Petit, M. Audran, J. Chromatogr. B 686 (1996) 3.
[25] ICH Q2B: ICH Harmonised Tripartite Guideline "Validation of Analytical Procedures: Methodology", November 1996.
[26] C. Le Guellec, M.L. Gaudet, S. Lamanetre, M. Breteau, Ther. Drug Monit. 19 (1997) 669.