Available online at www.sciencedirect.com
SCIENCE DIRECT®
Journal of Pharmaceutical and Biomedical Analysis
33 (2003) 1135–1142
JOURNAL OF PHARMACEUTICAL AND BIOMEDICAL ANALYSIS
www.elsevier.com/locate/jpba

Direct injection HPLC method for the determination of selected benzodiazepines in plasma using a Hisep column

C. Pistos, James T. Stewart *

Department of Pharmaceutical and Biomedical Sciences, College of Pharmacy, The University of Georgia, Athens, GA 30602-2352, USA

Received 19 September 2002; received in revised form 25 June 2003; accepted 18 July 2003

Abstract

A direct plasma injection HPLC method has been developed for the determination of selected benzodiazepines (nitrazepam, clobazam, oxazepam, lorazepam). The method uses an analytical hydrophobic shielded phase (Hisep) column equipped with a Hisep guard column, are easy to perform and requires 20 ul of a filtered plasma sample. The chromatographic run time is less than 15 min using a mobile phase of 15:85 v/v acetonitrile–0.18 M ammonium acetate pH 2.5. The method is good for 175 injections before replacement of the guard column. The method was linear in the range 0.5–18 ug ml^{-1} ($r > 0.99$, $n = 6$) for the analytes with R.S.D. less than 10.82%. Interday and intraday variability were found to be less than 14%. The limits of detection and quantitation were 0.16 ($s/n > 3$) and 0.5 ug ml^{-1} ($s/n > 10$), respectively, for each of the four benzodiazepines.

Keywords: High performance liquid chromatography; Direct injection; Benzodiazepines; Hisep column; Plasma

1. Introduction

A primary interest in the development of a direct injection technique in HPLC is the need for a simpler and faster analysis of a drug in biofluids [1]. Conventional reversed phase columns are not designed to handle large numbers of direct plasma injections since proteins can undergo denaturation and subsequent precipitation on the column can result in a clogged system. The restricted access media (RAM) columns are constructed in such a way that they can be useful for direct injection of spiked plasma or serum without any prior extraction of the drug [2]. The advantages of direct injection HPLC are: easier sample preparation, shorter analysis time, reduced cost of analysis, and excellent recovery of analytes. A disadvantage is a lack of sensitivity for analyzing low blood levels of some drug substances.

The direct injection method has been discussed in reviews by Wong [1] and Shihabi [3]. In the literature, the most widely used direct injection techniques include: (1) micellar chromatography [4], (2) column switching methods [5] and (3) the use of RAM columns [6–13]. Among these three techniques, the use of RAM columns is the simplest. Micellar chromatography involves an

* Corresponding author. Tel.: +1-706-542-4410; fax: +1-706-542-5358.
E-mail address: jstewart@rx.uga.edu (J.T. Stewart).

0731-7085/03/$ - see front matter
doi:10.1016/S0731-7085(03)00426-6

addition of a surface active agent to the mobile phase and although it improves column performance, it suffers from low resolution and sensitivity. Column switching is a more sensitive technique, but requires additional columns, pumps and switching valves.

The commercially available RAM columns include Pinkerton, also known as the internal surface reversed phase (ISRP), semi-permeable surface (SPS) and hydrophobic shielded phase (Hisep). All three columns are silica-based and designed to withstand direct biofluid injections. The stationary phases are prepared by modifying a silica surface with a hydrophobic bonded phase, which works like a partitioning phase. The hydrophobic bonded phase is then coated with a hydrophilic polymer, which, because of its pore size, can exclude large polymers such as proteins from interacting with the partitioning phase. When serum or plasma is injected onto RAM columns, the plasma proteins are size excluded by the outer hydrophilic polymer coating and flushed off with the solvent front. On the other hand, drug molecules, because of their smaller size, can penetrate the external polymer coating and interact with the partitioning phase.

In this paper, a direct plasma injection method for the determination of nitrazepam, clobazam, oxazepam and lorazepam (Fig. 1) is reported using a Hisep column. Benzodiazepines are among the most frequently prescribed drugs [14]. Their sedative-hypnotic, anxiolytic, tranquilizing and anticonvulsant effects are mediated by binding to a specific subtype of the $GABA_A$ receptor, i.e. the a1-type $GABA_A$ receptor, which is mainly expressed in cortical areas and in the thalamus of all vertebrates [15–17]. Its characterization in 1977 suggested the existence of endogenous ligands for the binding sites, which could be responsible for the physiological regulation of sleep, muscle tensions and anxiety [18].

Analytical methods previously reported for nitrazepam [19–22], clobazam [23–25], oxazepam [26–29] and lorazepam [30] include a number of HPLC assays. However, none of these references refer to the use of Hisep column. In addition, according to the manufacturer [31] a wide range of drug compounds have been studied under simple

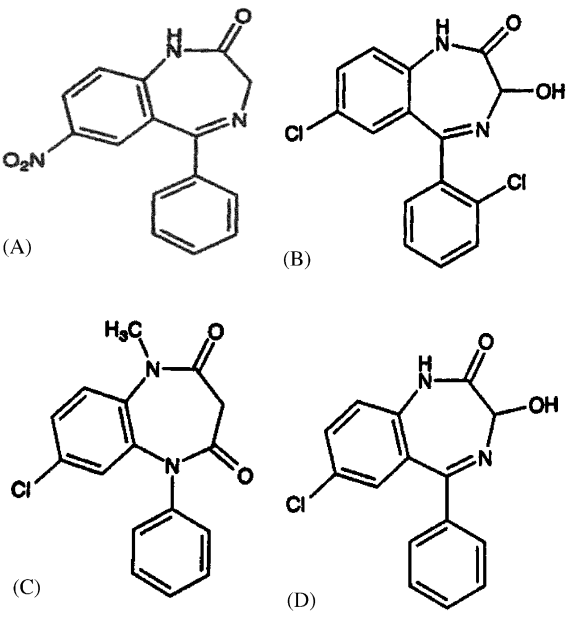


Fig. 1. Chemical structures of (A) nitrazepam, (B) clobazam, (C) oxazepam, (D) lorazepam.

chromatographic conditions but no report has been made for the application of benzodiazepines. A number of methods for the determination of some benzodiazepines and their metabolites were reported in the literature. Some methods used SPE and a combination of GC and FID [32], MS [33], nitrogen–phosphorus and electron capture detectors [34] with LODs of 50–100 ng ml^{-1}. The GC method with nitrogen–phosphorus and electron capture detector allows only toxicological determinations. Another method proposes the analysis of diazepam using a GC-mass selective detector after SPE with a LOD of 2.5 ng [35]. In these cases although the advantage of sensitivity, the method involves a time consuming SPE method and high cost instrumentation. Other methods used SPE and HPLC-DAD for the determination of nitrazepam [36], clobazam [37] and flunitrazepam with its metabolites [38] yielding LODs of 5, 15 and 200 ng ml^{-1}, respectively. The methods are more sensitive, but they also involve a SPE step. Another method proposes the use of fluorescence-TLC densitometric procedure for the determination of diazepam and oxazepam with LODs of 18 and 5 ng ml^{-1}, respectively [39]. A recent

literature survey indicated that there is still a need for pharmacokinetic and toxicokinetic studies of benzodiazepines. Most reported methods used liquid–liquid or SPE to isolate the analytes from plasma. In contrast, the direct injection method, which is described in this article, has the advantages of simplicity, rapidity and low cost for toxicological and pharmacological determinations. Our laboratory has previously reported direct injection analytical methods for phenylbutazone and NSAIs on SPS columns [40,41]. The direct injection method described herein requires no plasma sample clean up steps and should be applicable for the detection of ug levels of benzodiazepines in human plasma.

2. Experimental

2.1. Instrumentation

The HPLC system consisted of a Model 110 A pump (Beckman, Fullerton, CA), a Model 759 A variable wavelength UV/Vis detector (Applied Biosystems, Foster City, CA) and a Model 3394 A integrator (Hewlett Packard, Palo Alto, CA). A Valco Model C6W injection system (Valco Instrument Co, Houston, TX) equipped with a 20 ul loop was used for injection. The analytical column used was a Hisep (25 cm × 4.6 mm ID, 5 um particles). The Hisep column was protected with a guard column (Hisep 2 cm) containing the same packing material as the analytical column.

The Hisep shielded hydrophobic phase column selected for investigation in this present method can tolerate large numbers of small volume plasma injections. Feibush et al. [42], Gisch et al. [43], and Wong et al. [44] have reviewed the construction of the stationary phase in the Hisep column and its application to drugs. The material is silica-based covered with a polymer consisting of hydrophobic regions in a hydrophilic network. Small analytes, such as drugs, penetrate the hydrophilic network and are retained by the hydrophobic moieties. The hydrophilic network shields protein molecules from contact with the surface and the hydrophobic groups, and thus these molecules are not retained. The column has the ability to exclude proteins and avoid the column packing deterioration over a wide pH range (2.0–7.5) as the manufacturer proposes apart from other direct injection columns and techniques.

It is recommended by the manufacturer of the Hisep column that the mobile phase composition should contain $\leq 15\%$ organic modifier. This recommendation aids in the prevention of pressure buildup in the HPLC system. This restriction can also make it difficult to improve the elution of some classes of drugs, which are structurally featured by high hydrophobicity.

2.2. Reagents and chemicals

Nitrazepam was purchased from Hoffmann-La Roche Inc. Nutley, NJ and clobazam from Hoechst-Roussel Pharmaceuticals Inc., Somerville, NJ Lorazepam and oxazepam were USP reference standards (United States Pharmacopeia, Rockville, MD). Drug free plasma and filters (0.22 um, Millex®-GP) were obtained from Bioreclamation Inc. (Hicksville, NY) and Millipore Corporation (Bedford, MA), respectively. Acetonitrile and ammonium acetate were obtained from Fisher Scientific (Fair Lawn, NJ) and disposable 1 ml syringes from Becton Dickinson and Company (Franklin Lakes, NJ). Both the Hisep guard (Cat. No. 5-9639) and analytical column (Cat. No. 5-8919) were purchased from Supelco Inc. (Bellefonte, PA).

2.3. Chromatographic conditions

Separation of the four benzodiazepines was achieved on the Hisep column with the detector set at 254 nm and the column maintained at ambient temperature (23 ± 1 °C). The mobile phase was a mixture of 15:85 v/v acetonitrile–0.18 M ammonium acetate buffer pH 2.5 and the flow rate was maintained at 2 ml min^{-1}.

2.4. Sample preparation

A combined stock solution containing 500 ug ml^{-1} of nitrazepam, clobazam, oxazepam and lorazepam was prepared in acetonitrile and stored at 4 °C. A second dilution was made to prepare a

combined stock solution containing 100 ug ml^{-1} in water. Appropriate aliquots of this solution were then added to individual 1 ml volumetric tubes, and filtered plasma added to volume to give concentrations in the 0.5–18 ug ml^{-1} range. Each tube was vortexed for 30 s and 20 ul of the filtered plasma sample was injected onto the HPLC column.

3. Results and discussion

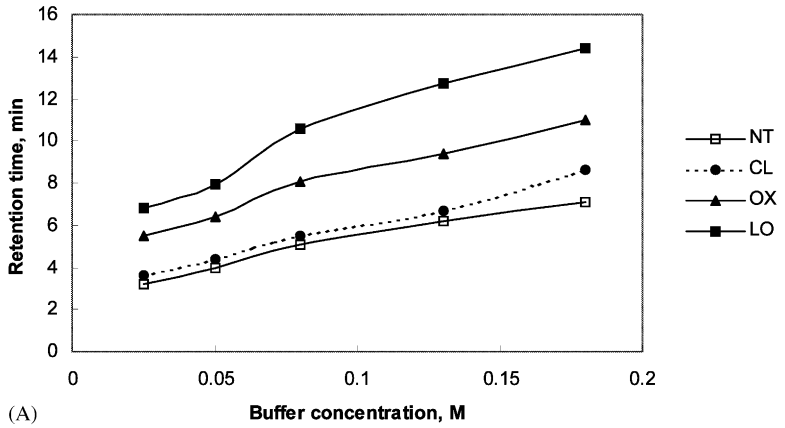
The use of acetonitrile as a dilution solvent for the benzodiazepine drug in plasma led to a lack of reproducibility during injection onto the analytical column, something, which was not observed by using water as the dilution media. Thus, it was necessary to make the second dilution in water.

During our preliminary experiments, we tried several combinations of the mobile phase composition and pH in order to obtain the optimum separation. Thus, we examined buffer concentrations at 0.025, 0.05, 0.08, 0.13 and 0.18 M ammonium acetate, mobile phase pH at 2.5, 5, and 7.25, and acetonitrile percentages at 5, 7.5, 10, 12.5, and 15%. In the first case, it was observed that ammonium acetate concentration in the mobile phase affected retention time of the analytes. It was found that a 0.18 M buffer concentration gave the best separation in the shortest time since at lower concentration of the buffer, nitrazepam and clobazam peaks overlapped or were interfered with by the protein peaks (Fig. 2A). In the second case, pH affected retention time and separation. The result was an overlapping between the analyte peaks, which consequently led to a separation failure (Fig. 2B). The retention

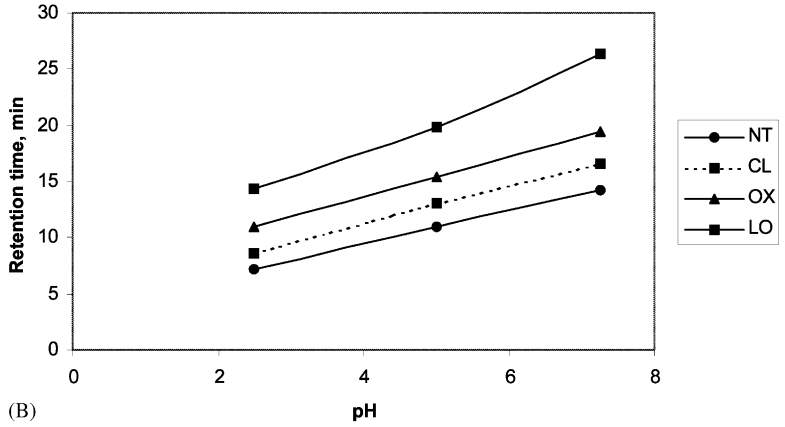
Table 1
Intraday (n = 3) and interday (n = 9) accuracy and precision data for nitrazepam, clobazam, oxazepam and lorazepam in spiked plasma samples[a]

Concentration added (ug ml^{-1})	Concentration found (ug ml^{-1})	% Error	% R.S.D.	Concentration found (ug ml^{-1})	% Error	% R.S.D.
	Nitrazepam			Clobazam		
Intraday ($n = 3$)						
1.5	1.3±0.1	11.3	4.5	1.3±0	11.3	0
8	6.8±0.2	15.3	2.9	7.2±1.0	9.6	13.8
15	13.2±1	11.9	7.6	13.5±0.8	9.9	5.9
	Oxazepam			Lorazepam		
1.5	1.4±0.1	6.6	4.3	1.3±0	16.7	0
8	7.1±1.0	11.4	14	7.2±1.0	10	13.9
15	13.2±0.8	12	6	12.9±0.5	14	3.9
	Nitrazepam			Clobazam		
Interday ($n = 9$)						
1.5	1.5±0.1	0.7	6.7	1.5±0.1	3.3	3.4
8	8.0±0.3	0.5	3.8	7.8±0.2	2.5	2.6
15	13.8±1.0	8.12	7.3	13.7±1.0	8.7	7.3
	Oxazepam			Lorazepam		
1.5	1.4±0.1	8.6	4.4	1.4±0.1	4.7	4.9
8	7.9±0.1	1.3	1.3	7.9±0.1	1.6	1.3
15	13.6±0.9	9.5	6.6	13.5±1.5	9.9	11.1

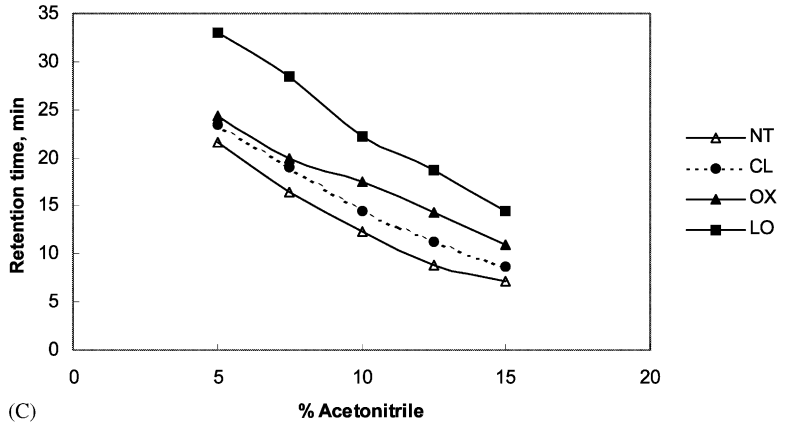
[a] Stock solutions were stored up to 90 days. No significant difference was observed between the initial stock and a freshly prepared stock of the standard concentration.



(A)



(B)



(C)

Fig. 2. Effect of (A) buffer concentration, (B) pH and (C) % ACN on the retention time of nitrazepam (NT), clobazam (CL), oxazepam (OX) and lorazepam (LO).

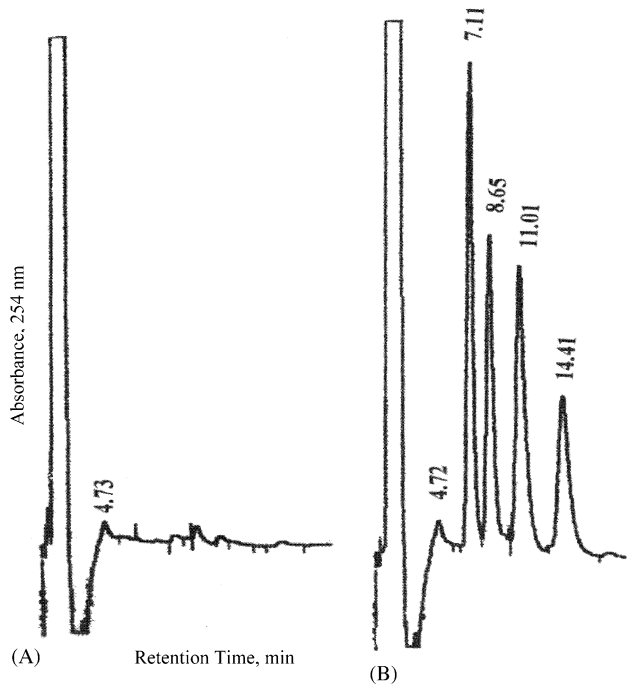


Fig. 3. Typical chromatograms of (A) blank plasma and (B) spiked plasma containing 18 ug ml^{-1} each of nitrazepam (7.11 min), clobazam (8.65 min), oxazepam (11.01 min) and lorazepam (14.41 min). HPLC conditions: Hisep column; mobile phase was 15:85 acetonitrile–0.18 M ammonium acetate buffer pH 2.5; flow rate, 2 ml min^{-1}; detection at 254 nm; injection volume, 20 ul; ambient temperature (23 °C). Resolution (R), is 1.5 between nitrazepam and clobazam, 2.4 between oxazepam and clobazam and 1.7 between lorazepam and oxazepam.

times for all the drugs were longer as the percentage of acetonitrile was decreased (Fig. 2C).

The best separation of nitrazepam, clobazam, oxazepam and lorazepam on the Hisep column was achieved using a mobile phase of 15:85 v/v acetonitrile–0.18 M ammonium acetate buffer pH 2.5, with retention times of 7.11, 8.65, 11.01 and 14.41 min, respectively (Fig. 3). Besides these particular benzodiazepines, four other benzodiazepines were also found to have a very similar response on the Hisep column. Using the same mobile phase composition and chromatographic condition as previously described in this paper, temazepam, diazepam, flurazepam and clonazepam were eluted at 8.66, 3.23, 1.41 and 14.06 min, respectively. The clobazam and lorazepam peaks showed overlap and interfered with the peaks of temazepam and clonazepam, respectively. Additionally, diazepam and flurazepam peaks were interfered by the endogenous plasma peaks. Although there is an ability to change the composition of the mobile phase and separate these drugs, it was not possible to concurrently determine all eight of them in a single injection. It is more feasible to apply this method to determine each drug separately or in certain combinations.

Calibration curves were prepared on the Hisep column for a nitrazepam, clobazam, oxazepam and lorazepam mixture in plasma at 0.5–18 ug ml^{-1} levels. Linear regression analysis for each analyte was performed using concentration versus peak height response. Typical correlation coefficient, slope and intercept for nitrazepam were 0.999, 6.86 and 1.14, for clobazam 0.99, 4.32, 1.52, for oxazepam 0.999, 3.93, 0.79 ($n = 6$) and for lorazepam 0.99, 2.2, 1.1 ($n = 6$), respectively.

An internal standard was unnecessary in this direct injection plasma assay since there were no sample extraction steps. Intraday and interday accuracy and precision data for the direct injection method using spiked drug samples are shown in Table 1. Intraday data ($n = 3$) were based on triplicate injections and interday data ($n = 9$) were based on triplicate data over 3 days, respectively.

Absolute recoveries of nitrazepam, clobazam, oxazepam and lorazepam on the Hisep column were determined to be 99.5 ± 3.75, 97.5 ± 2.5, 98.8 ± 1.3 and $98.4 \pm 1.3\%$, respectively ($n = 9$), comparing peak height response of known analyte concentrations in plasma versus water.

The injection volume of plasma was restricted to 20 ul to enhance Hisep column life. There was a significant pressure build-up over time in the HPLC system due to the protein clogging despite the use of a guard column and filtered plasma samples. Approximately 175 total plasma injections were made before the system pressure reached 3500 psi. Beyond 175 injections, the performance of the Hisep analytical column did not yield any significant difference in its reproducibility or system pressure when the Hisep guard column was replaced.

4. Conclusions

The direct injection method for selected benzodiazepines (nitrazepam, clobazam, oxazepam, lorazepam) in plasma using the Hisep column is easy to perform, uses small sample volumes, requires no sample pretreatment steps and possesses the necessary sensitivity and reproducibility to be of use in pharmacokinetic and toxicokinetic studies of these analytes. In contrast to other direct injection columns, the Hisep column seems to be valuable in approaching its advertisement indications. The back pressure of the system increases significantly only after 175 direct plasma injections. Laboratories running a large number of samples each day may find the use of the Hisep column to be too expensive and wasteful with unnecessary down time for cleaning of column frits or changing the Hisep guard column. It can be a powerful tool when it is desirable to have a simple, rapid and accurate determination of overdose levels for a wide range of drugs in the benzodiazepine class.

References

[1] S.H.Y. Wong, J. Pharm. Biomed. Anal. 7 (1989) 1011–1032.
[2] T.C. Pinkerton, J. Chromatogr. 544 (1991) 13–23.
[3] Z.K. Shihabi, J. Liquid Chromatogr. 11 (1988) 1579–1593.
[4] M.J. Koenigbauer, J. Chromatogr. Biomed. Appl. 531 (1990) 79–99.
[5] C.F. Pilar, H.H. Rosa, S.C. Adela, J. Chromatogr. Biomed. Appl. 619 (1993) 177–190.
[6] J.D. Brewster, A.R. Lightfield, R.A. Barford, J. Chromatogr. 598 (1992) 23–31.
[7] J. Ma, C.L. Liu, P.L. Zhu, Z.P. Jia, L.T. Xu, R. Wang, J. Chromatogr. B Anal. Technol. Biomed. Life Sci. 772 (2002) 197–204.
[8] R. Ueno, T. Aoki, J. Chromatogr. B Biomed. Appl. 682 (1996) 179–181.
[9] K. Uno, I. Maeda, J. Chromatogr. B Biomed. Appl. 663 (1995) 177–180.
[10] A.M. Andeejani, H. Hughes, D.M. Feuchuk, H.Y. Aboul-Enein, Biomed. Chromatogr. 8 (1994) 26–28.
[11] R. Ueno, K. Uno, T. Aoki, J. Chromatogr. 573 (1992) 333–335.
[12] E. Riva, R. Merati, L. Cavenaghi, J. Chromatogr. 553 (1991) 35–40.
[13] M.R. Lockemeyer, C.V. Smith, J. Chromatogr. 532 (1990) 162–167.
[14] H. Coper, H. Helmchen, Tranquilizer, in: W. Forth, D. Henchler, W. Rummel, K. Starke (Eds.), Allgemeine und Spezielle Pharmakologie und Toxikologie, Spektrum Akademischer Verlag GmbH, Heidelberg, 1996, pp. 291–295.
[15] H. Möhler, T. Okada, Science 198 (1977) 849–851.
[16] R.F. Squires, C. Braestrup, Nature 266 (1977) 732–734.
[17] U. Rudolph, F. Crestani, D. Benke, I. Brünig, J.A. Benson, J.M. Fritschy, J.R. Martin, H. Bluethmann, H. Möhler, Nature 401 (1999) 796–800.
[18] D. Kavvadias, A.A. Abou-Mandour, F.-C. Czygan, H. Beckmann, P. Sand, P. Riederer, P. Schreier, Biochem. Biophys. Res. Commun. 269 (2000) 290–295.
[19] M. Wilhelm, H.J. Battista, D. Obendorf, J. Anal. Toxicol. 25 (2001) 250–257.
[20] K. Jinno, M. Taniguchi, M. Hayashida, J. Pharm. Biomed. Anal. 17 (1998) 1081–1091.
[21] M. Kleinschnitz, M. Herderich, P. Schreier, J. Chromtogr. B 676 (1996) 61–67.
[22] M.D. Robertson, O.H. Drummer, J. Chromatogr. B 667 (1995) 179–184.
[23] A. Bolner, F. Tagliaro, A. Lomeo, J. Chromatogr. B 750 (2001) 177–180.
[24] P.K. Kunicki, J. Chromatogr. B 750 (2001) 41–49.

[25] J. Knapp, P. Boknik, H.G. Gumbinger, B. Linck, H. Luss, F.U. Muller, W. Schmitz, U. Vahlensieck, J. Neumann, J. Chromatogr. Sci. 375 (1999) 145–149.
[26] A. El-Mahjoub, C. Staub, J. Chromatogr. B 742 (2000) 381–390.
[27] H. Levert, P. Odou, H. Robert, Biomed. Chromatogr. 16 (2002) 19–24.
[28] A. El Mahjoub, C. Staub, Forensic Sci. Int. 123 (2001) 17–25.
[29] A. El Mahjoub, C. Staub, J. Pharm. Biomed. Anal. 23 (2000) 447–458.
[30] H. Kanazawa, Y. Kunito, Y. Matshushima, S. Okubo, F. Mashige, J. Chromatogr. A 871 (2000) 181–188.
[31] Supelco's application note T 397145A.
[32] M.P. Quaglio, A.M. Bellini, L. Minozzi, Farmaco 47 (1992) 799–809.
[33] U. Staerk, W.R. Kulpmann, J. Chromatogr. B 745 (2000) 399–411.
[34] Y. Gaillard, J.P. Gay-Montchamp, M. Ollagnier, J. Chromatogr. 622 (1993) 197–208.
[35] M.W. van Hout, W.M. van Egmond, J.P. Franke, R.A. de Zeeuw, G.J. de Jong, J. Chromatogr. B 766 (2002) 37–45.
[36] K.K. Akerman, J. Jolkkonen, M. Parviainen, I. Penttila, Clin. Chem. 42 (1996) 1412–1416.
[37] K.K. Akerman, Scand. J. Clin. Lab. Invest. 56 (1996) 609–614.
[38] W. He, N. Parissis, J. Pharm. Biomed. Anal. 16 (1997) 707–715.
[39] S.R. Sun, J. Pharm. Sci. 67 (1978) 1413–1415.
[40] A. Haque, J.T. Stewart, J. Pharm. Biomed. Anal. 16 (1997) 287–293.
[41] A. Haque, J.T. Stewart, Biomed. Chromatogr. 13 (1999) 51–56.
[42] B. Feibush, C. Santasania, J. Chromatogr. 54 (1991) 441–449.
[43] D.J. Gisch, B.T. Hunter, B. Feibush, J. Chromatogr. 433 (1988) 264–268.
[44] S.H.Y. Wong, L.A. Brett, A.C. Larson, J. Liquid Chromatogr. 11 (1988) 2039–2049.