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Journal of Chromatography B, 704 (1997) 251–258

JOURNAL OF CHROMATOGRAPHY B

Simple high-performance liquid chromatographic column-switching technique for the on-line immunoaffinity extraction and analysis of flunitrazepam and its main metabolites in urine

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Received 25 October 1996; received in revised form 5 August 1997; accepted 21 August 1997

Abstract

A sensitive, simple and rapid method without sample pretreatment is presented for the simultaneous determination of flunitrazepam and its main metabolites (norflunitrazepam, 7-amino- and 7-acetamidoflunitrazepam) in urine. The single-step procedure is based on a column-switching technique which uses an immobilized antibody in an extraction column following concentration on a precolumn and separation on an analytical column. UV detection was performed at 254 nm. The reusability of the antibody exceeds 88 runs and a complete analysis was performed in less than 40 min. The method shows coefficients of variation below 9.9% and rates of recovery greater than 92% tested at the level of 50 ng/ml urine. The limit of detection was below 2 ng/ml urine for the four compounds. © 1997 Elsevier Science B.V.

Keywords: Flunitrazepam; Benzodiazepines

1. Introduction

In order to separate certain molecules from biological matrices, immunoaffinity techniques are superior to traditional liquid- or solid-phase extraction methods [1,8,11–14,17]. Antibody-mediated extractions reveal a high selectivity and therefore permit a very high clean-up efficiency.

In recent years, a growing number of immunoaffinity extraction techniques have been described in the literature, especially in the field of veterinary and doping analysis [5–8]. Such immunoaffinity extractions can be performed in the off-line and in the on-line mode, but only the on-line technique offers the following advantages: direct sample injection without sample pretreatment, avoiding of extraction losses during the evaporation and reconstitution steps, better reproducibility, reduced contamination danger and no contact with toxic solvents [1,9–11,17].

In the present paper we describe a simple, rapid and sensitive method for the quantitative determination of flunitrazepam and its main metabolites (7-amino-, 7-acetamidoflunitrazepam and norflunitrazepam) in urine. Flunitrazepam belongs to the 1,4-benzodiazepine class and is widely used as a potent hypnotic drug; but its abuse has also greatly increased over the last years [2,4,15,16].

The single-step procedure is based on a simple HPLC column-switching technique, which uses an

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0378-4347/97/$17.00
PII S0378-4347(97)00429-5

immobilized antibody in an immunoextraction column (IAC), a preconcentration column (PC) and an analytical column (AC). UV detection was performed at 254 nm. In contrast to similar techniques published only one tandem switching valve will be necessary to realise this switching technique [1,3].

No on-line HPLC technique for benzodiazepines using an antibody-mediated extraction could be found in the literature.

2. Experimental

2.1. Materials

HPLC-grade solvents (acetonitrile and methanol) and p.a. grade chemicals (potassium dihydrogen phosphate, sodium azide, sodium chloride) and p.a. grade orthophosphoric acid (85%) were purchased from Merck (Darmstadt, Germany). HPLC-grade water was prepared using a Milli-Q purification system (Millipore, Bedford, MA, USA).

Flunitrazepam, norflunitrazepam, 7-amino- and 7-acetamidoflunitrazepam were kindly provided by Hoffmann-La Roche (Basle, Switzerland).

Stock solutions of each reference standard were prepared at a concentration of 1 mg/ml methanol and stored at 4°C in the dark. The methanolic working solutions contained all four standards at a concentration of 10 and 1 ng/ml.

The polyclonal antibody, raised against benzodiazepines, was kindly provided by Boehringer Mannheim (Germany). The properties of the antibody are described in detail elsewhere [17]. Following the manufacturer's recommended procedure, 1 mg of the antibody was immobilized on glutardialdehyde-activated affinity sorbent (Boehringer Mannheim). The immunosorbent obtained had a capacity of 1200 ng flunitrazepam.

The mobile phase was a mixture of acetonitrile–0.05 *M* potassium phosphate buffer, pH 2.0 (36:64, v/v). The pH of the buffer was adjusted by addition of orthophosphoric acid under control of a pH meter.

2.2. Apparatus

The scheme of the HPLC on-line system is shown in Fig. 1. Three HPLC pumps (model 2150, LKB, Bromma, Sweden), a tandem switching valve (Motorventil H, Besta, Heidelberg, Germany) which included two Rheodyne model 7000 two-position six-port switching valves and an UV detector (model 2150, LKB, Bromma) were used. The injector (model 7125, Rheodyne, Cotati, CA, USA) was equipped with a 2.0-ml loop, which was made by using 1/16-in. stainless steel tubing of 1 mm diameter. The mobile phase delivered by pump 1 (P1) at a flow-rate of 0.5 ml/min was HPLC-grade water, pump 2 (P2) delivered methanol–water (90:10, v/v) at a flow-rate of 0.05 ml/min and pump 3 (P3) delivered acetonitrile–0.05 *M* phosphate buffer, pH 2.0 (36:64, v/v) at a flow-rate of 1.0 ml/min. The immunoaffinity column (IAC) was a 20×4-mm HPLC cartridge, filled with immunosorbent (200 μl) and equipped with 5-μm stainless steel filters and teflon rings for immobilisation in the cartridge holder (Müller, Fridolfing, Germany). The preconcentration column (PC) was a 20×4 mm HPLC cartridge, filled with 5-μm particles of LiChrospher 60 RP-Select B (Merck) under industrial conditions (Müller). The analytical column (AC) was a LiChrospher 60 RP-Select B cartridge (25×4.6 mm, 5 μm), purchased from Merck. All capillary connections were made with 1/16-in. Peek capillaries of 0.17 mm diameter. The dead-volume free T-connector (Valco Instruments) was purchased from Müller. The centrifuge used was a model 1306 from Hettrich (Tuttlingen,

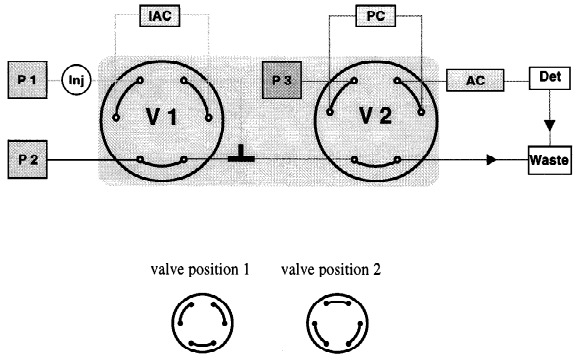

Fig. 1. Set-up of the immunoaffinity-LC on-line system described in Section 2. The valves indicated in the figure are components of a tandem unit and can only be switched simultaneously. Two positions of the tandem unit are possible which allow selection of the operating steps summarized in Table 1. The valves (V1, V2) shown in the figure have the position 1.

Germany) and the pH meter was a model 70 from Knick (Berlin, Germany).

2.3. Procedure

The on-line analysis of samples was performed with the system shown in Fig. 1 and the three sequential steps shown in Table 1. In Step 1 the centrifuged and diluted urine samples were loaded into the loop and injected into the system. During the first 10 min (tandem valve position 1) the sample was pumped through the IAC by P1 at a flow-rate of 0.5 ml/min, followed by water as washing solvent. P3 delivered the mobile phase through the PC and the AC. After switching the tandem valve into position 2 (step 2) the 90% methanol was pumped by P2 through the IAC at a flow-rate of 0.05 ml/min for 10 min, allowing the quantitative desorption of the analytes. P1 was used in this step to dilute the IAC elution mixture with water to a final methanol concentration below 10% (T-piece) in order to trap the analytes on the PC. In step 3 (tandem valve back in position 1) the mobile phase eluted the analytes from the PC following separation on the AC and detection/integration. In this state the IAC was regenerated by water and prepared for the next injection. After 40 min the system was ready for another analysis.

Prior to on-line analysis the urine samples were centrifuged for 10 min at 12 000 rpm. The supernatant (250 μl) was diluted with 750 μl of HPLC grade water and the mixture (1 ml) was injected into the system.

When not in use the immunocolumn was stored in a solution of sodium azide (0.1%) and sodium chloride (0.9%) in water at room temperature.

2.4. Calculations

The concentrations were calculated by comparing the peakheights from samples to the corresponding standards containing known amounts of flunitrazepam and the metabolites. Six standards were used to produce a standard curve for each compound. Linear regression analysis of the standard curves were made using the software Kaleidagraph on an Apple Macintosh Computer.

The percent recoveries were calculated by injecting blank urine samples spiked with known amounts of the reference standards. The resulting peak heights were compared with those obtained from the pure standards injected after connecting the injector directly with the analytical column and using a 20-μl loop instead of the 2-ml loop.

3. Results and discussion

3.1. Immunoaffinity extraction and reconcentration of the analytes.

In the first step of the method development the flow-rate of P1 was optimized in order to retain the

Table 1
Sequential steps of the on-line procedure

Step	Time interval (min)	Valve position		Analytical operation
		V1	V2	
1	0–10	1	1	Injection of the sample onto IAC. Adsorption of the analytes and removal of matrix constituents by subsequent washing with water (P1).
2	10–20	2	2	Desorption of the analytes from IAC with methanol–water 90:10 (P2), subsequent dilution with water (P1) and trapping of the analytes on the PC.
3	20–40	1	1	Transfer of the enriched analytes onto AC and chromatographic separation by the mobile phase (P3). Reequilibration of IAC by water (P1).

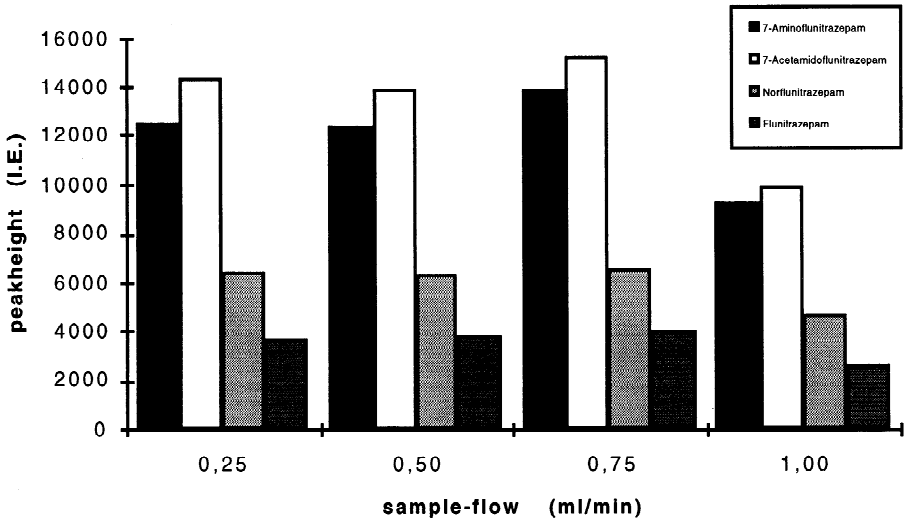

Fig. 2. IAC adsorption kinetics of flunitrazepam and metabolites as a function of the flow-rate of the sample.

analytes on the IAC. In Fig. 2 the adsorption kinetics of flunitrazepam and metabolites on the IAC are shown. No analyte losses occurred up to a flow-rate of 0.75 ml/min. Therefore a flow-rate of 0.5 ml/min for P1 was chosen for the sample transport through the IAC.

The desorption of the analytes from the IAC was performed with 90% methanol (delivered by P2), which is well documented in the literature as a potent elution solvent for immunoaffinity columns. In order to reconcentrate the desorbed analytes on the PC the methanol content of the desorption mixture was lowered by dilution in the T-piece where the eluents delivered by P1 and P2 were combined. Setting the flow-rate of P2 at 0.05 ml/min, the difference of the flow-rates of P1 and P2 led to a decrease of the methanol content from 90 to 8.2% allowing the adsorption of the analytes on the PC. Therefore we studied the desorption kinetics of flunitrazepam and its metabolites at a flow-rate of 0.05 ml/min for P2. In Fig. 3 it can be seen that 9 min were necessary for quantitative desorption of the analytes under these

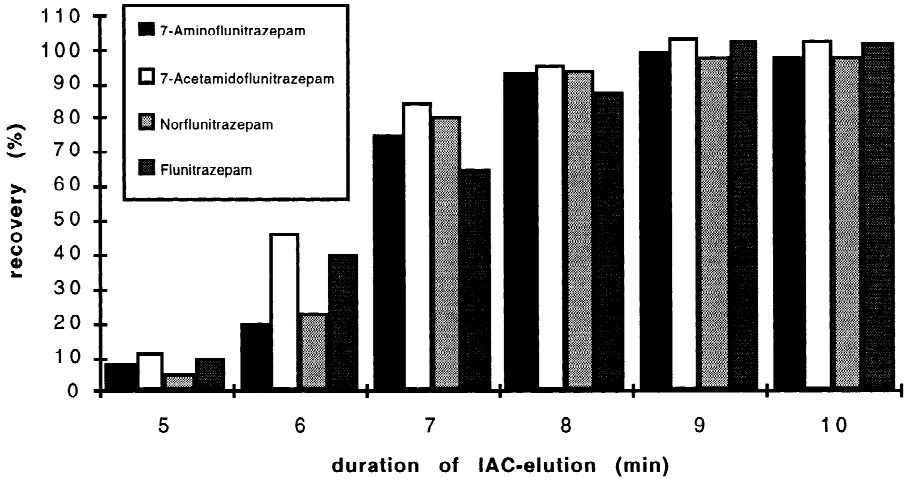

Fig. 3. IAC desorption kinetics of flunitrazepam and metabolites with 90% methanol as elution solvent at a flow-rate of 0.05 ml/min.

conditions. So we chose an interval of 10 min for this step.

The capacity of the IAC applied was determined by injecting a high amount of flunitrazepam (5000 ng/ml water). Fig. 4 demonstrates that there was an initial strong decrease of the capacity during the first injections. After the 10th injection the capacity reached a constant level at about 1200 ng and did not show a significant loss up to the 88th injection.

The efficient clean-up procedure of the immunoaffinity technique applied is illustrated by Fig. 5: the chromatogram shows an extracted urine sample where no interfering compounds were present. In Fig. 6 the extract of a spiked urine sample is shown: each peak corresponds to a urine concentration of 50 ng/ml (12.5 ng/injection).

3.2. HPLC separation.

The isocratic HPLC eluent which was developed for the separation of various benzodiazepines and metabolites in a previous study included a phosphate buffer adjusted at pH 2.0, because at higher pH values an incomplete separation of 7-amino- and 7-acetamidoflunitrazepam has been observed on the LiChrospher RP-Select B phase [2,18]. The pH of 2.0 in the mobile HPLC phase did not lead to any decrease in the performance of the column over a period of 5 months.

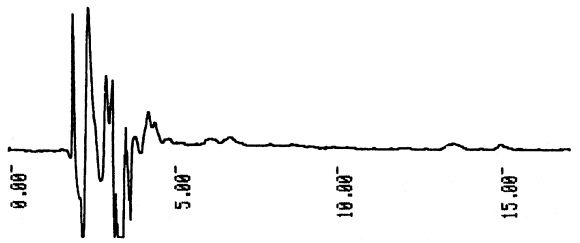

Fig. 5. Chromatogram of a blank urine extract. The peaks in the front area result from the column-switching procedure (cycle 2 into cycle 3).

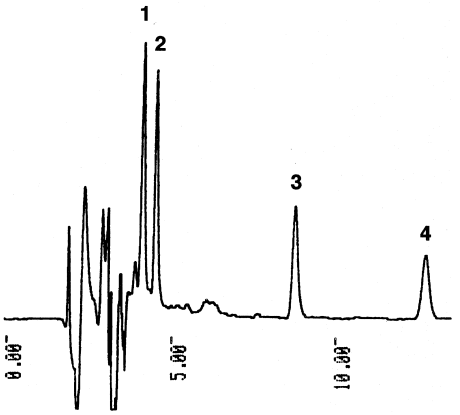

Fig. 6. Chromatogram of an extracted standard mixture (1, 7-aminoflunitrazepam; 2, 7-acetamidoflunitrazepam; 3, norflunitrazepam; 4, flunitrazepam). Each peak represents a concentration of 50 ng/ml urine (12.5 ng injected).

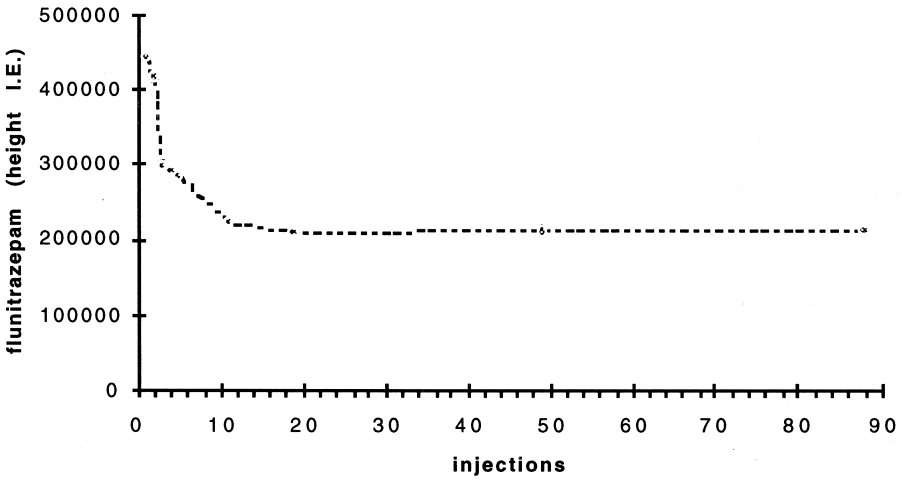

Fig. 4. Capacity of the IAC as a function of the number of analyses performed.

3.3. Method validation and application.

Linearity was studied in the range from 5 to 1000 ng in 1-ml aliquots of diluted urine samples. Prior to dilution the urine samples were spiked with the corresponding amounts of flunitrazepam and metabolites. All compounds gave linear relationships over the whole range tested with coefficients of correlation greater than 0.999 (Fig. 7).

As a consequence of the high selectivity of the antibody and the elimination of critical off-line extraction steps like evaporation or reconstitution, the immunoaffinity on-line technique revealed excellent recoveries as shown in Table 2.

Precision and accuracy data are summarized in Table 3: the coefficients of variation did not exceed 7.1% for the intra-assay and 9.9% for the day-to-day assay tested at a concentration of 50 ng/ml urine. The limit of detection, estimated at a signal-to-noise ratio of 3:1 was below 2 ng/ml urine for all compounds.

Table 2
Recovery data (%) for flunitrazepam and metabolites after immunoaffinity extraction and on-line analysis (mean of four determinations)

Compound	12.5 ng injected	250 ng injected
Flunitrazepam	98	94
Norflunitrazepam	96	94
7-Acetamidoflunitrazepam	92	98
7-Aminoflunitrazepam	100	98

Fig. 8 shows an analyzed urine sample of a volunteer, collected 12.8 h after intake of a 2-mg oral dose of flunitrazepam. Typically for therapeutic doses the 7-aminometabolites, but no unchanged flunitrazepam or norflunitrazepam, are detectable in urine at this time point.

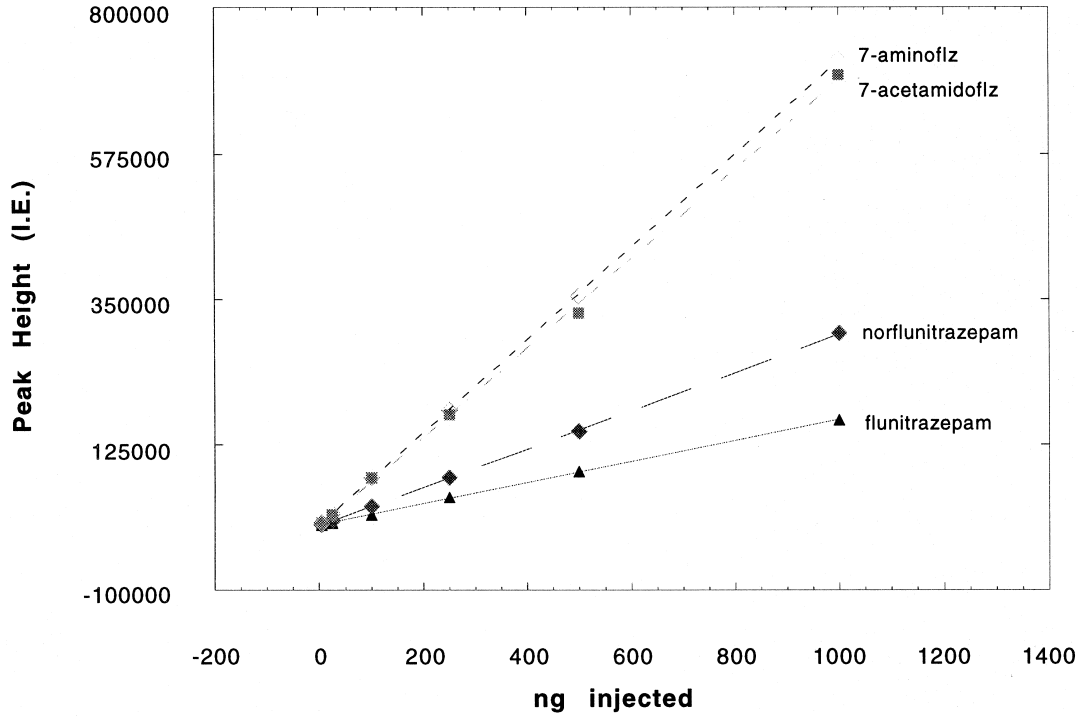

Fig. 7. Linear regression analysis for flunitrazepam and metabolites in the range from 5 to 1000 ng injected. The corresponding equations and coefficients of correlations are: $y=693+164x$, $r^2=0.9993$ (flunitrazepam); $y=593+297x$, $r^2=0.9995$ (norflunitrazepam); $y=1154+690x$, $r^2=0.9995$ (7-acetamidoflunitrazepam); $y=169+718x$, $r^2=0.9996$ (7-aminoflunitrazepam).

Table 3
Precision and accuracy data for flunitrazepam and metabolites

Compound	Intra-assay ($n=5$)			Inter-assay ($n=10$)		
	Amount (ng)		C.V. (%)	Amount (ng)		C.V. (%)
	Added	Found		Added	Found	
Flunitrazepam	12.5	11.7	3.3	12.5	12.7	7.6
Norflunitrazepam	12.5	11.8	1.9	12.5	12.6	9.9
7-Acetamidoflunitrazepam	12.5	10.8	3.7	12.5	12.0	8.4
7-Aminoflunitrazepam	12.5	13.0	7.1	12.5	13.0	5.1

4. Conclusions

We have shown that the analytical system described in this paper is able to give fast, quantitative and reliable information for the benzodiazepine drug flunitrazepam and its major metabolites in urine. The developed column-switching technique has a simple construction and, with only three operating steps, it is easy to perform. The on-line system permits the direct injection and analysis of the sample without pretreatment. The immobilized antibody allows an efficient sample clean-up resulting in highly purified extracts and shows a reusability of at least 88 applications. The system can be fully automated and it can be extended to other benzodiazepines. With suitable antibodies the transfer to other classes of drugs will be possible. This opens new routes in clinical or forensic case work.

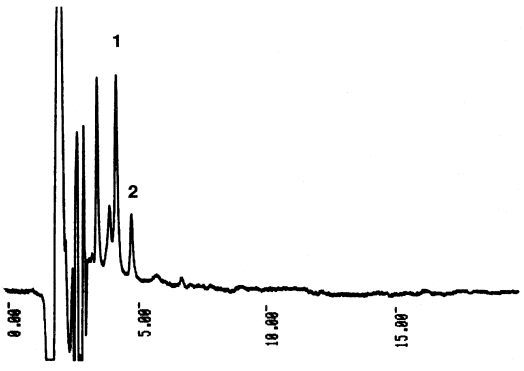

Fig. 8. Chromatogram of a volunteer's urine sample collected 12.8 h after intake of an 2-mg oral dose of flunitrazepam (1, 7-aminoflunitrazepam, 31.2 ng/ml; 2, 7-acetamidoflunitrazepam, 11.6 ng/ml).

Acknowledgements

This work was supported by Boehringer Mannheim, which kindly provided the antibody and the affinity sorbent materials for immobilisation.

References

[1] E. Davoli, R. Fanelli, R. Bagnati, Anal. Chem. 65 (1993) 2679.
[2] I. Deinl, Ph.D. Thesis, Ludwig-Maximilians-Universität München, Munich, 1996.
[3] T.D. Eller, K. Walle, T. Walle, J. Chromatogr. 612 (1993) 320.
[4] I.M. McIntyre, M.L. Syrjanen, K. Crump, S. Horomidis, A.W. Peace, J. Anal. Toxicol. 17 (1993) 202.
[5] A. Farjam, G.J. de Jong, R.W. Frei, U.A.Th. Brinkman, W. Haasnot, A.R.M. Hamers, R. Schilt, F.A. Huf, J. Chromatogr. 432 (1988) 419.
[6] C. van de Water, D. Tebbal, N. Haagsma, J. Chromatogr. 478 (1989) 205.
[7] L.A. van Ginkel, R.W. Stephany, H.J. van Rossum, H. van Blitterswijk, P.W. Zoontjes, R.C.M. Hoojschuur, J. Zuydendorp, J. Chromatogr. 489 (1989) 95.
[8] L.A. van Ginkel, J. Chromatogr. 564 (1991) 363.
[9] W. Haasnot, R. Schilt, A.R.M. Hamers, F.A. Huf, A. Farjam, R.W. Frei, U.A.Th. Brinkman, J. Chromatogr. 489 (1989) 157.
[10] S.E. Katz, M. Siewierski, J. Chromatogr. 624 (1992) 403.
[11] G.S. Rule, J.D. Henion, J. Chromatogr. 582 (1992) 103.
[12] M. Sharman, J. Gilbert, J. Chromatogr. 543 (1991) 220.
[13] C. van de Water, N. Haagsma, J. Chromatogr. 411 (1987) 415.

[14] G.M. Ware, P.P. Umrigar, A.S. Carman, S.S. Kuan, Anal. Lett. 27 (1994) 693.
[15] W. Loosen, Sucht 2 (1994) 127.
[16] U. Schwabe, D. Paffrath, Arzneiverordnungsreport '95, Gustav Fischer Verlag, Stuttgart, 1996.
[17] C. Franzelius, I. Ackermann, I. Deinl, L. Angermaier, G. Machbert, submitted for publication to J. Anal. Toxicol.
[18] I. Deinl, G. Mahr, L. von Meyer, submitted for publication to J. Anal. Toxicol.