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High-performance liquid chromatographic method for the determination of benzodiazepines in plasma or serum using the column-switching technique

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Abstract

A column-switching high-performance liquid chromatographic method for the simultaneous determination of five frequently prescribed benzodiazepines: clonazepam, diazepam, flunitrazepam, midazolam and oxazepam was developed. A 50-μl plasma sample was directly injected into a BioTrap 500 MS (hydrophobic polymer) column. After a washing step with a mixture of phosphate buffer and acetonitrile, the retained benzodiazepines were back-flushed into a reversed-phase (LiChrospher Select B C_8) column with a mobile phase of acetonitrile–phosphate buffer. The method showed excellent linearity from 50 to 1000 ng/ml for clonazepam, flunitrazepam and midazolam and from 50 to 5000 ng/ml for diazepam and oxazepam. The recoveries were around 98% for all the benzodiazepines studied. The relative standard deviation for between- and within-day assay was <20% for low concentrations close to the values of the limit of quantification and <4% for high concentrations. The procedure described is relatively simple and rapid because no off-line manipulation of the sample is required: the total analysis time is approximately 30 min. © 2000 Elsevier Science B.V. All rights reserved.

Keywords: Column switching; Benzodiazepines

1. Introduction

The rapid and sensitive analysis of benzodiazepines (BZDs) is an area of increasing interest, because these compounds have become more and more the subject of abuse [1–4].

Many analytical procedures have been developed for the quantification of benzodiazepines in biological matrices. Common methods are gas chromatography (GC) and high-performance liquid chromatography (HPLC) [5].

Gas chromatography–mass spectrometry (GC–MS) has been traditionally recommended for the analysis of drugs in biological sample because of the high sensitivity achieved [6–10].

In order to simplify the whole analytical procedure different methods have been proposed for automation.

The chromatographic theory and method development behind column-switching or on-line pre-concentration techniques have been well reviewed and described [11,12].

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This work describes a column-switching technique in which the plasma samples are directly injected after centrifugation, into a BioTrap 500 MS column (extraction column) and washed with a mobile phase consisted of 30 m*M* phosphate buffer (pH 7.2)–acetonitrile (94:6, v/v). This on-line step ensures an extremely clean chromatographic trace. The retained drugs are then eluted onto a C_8 reversed-phase analytical column for determination. The column-switching technique offers several advantages: direct injection of untreated biological fluids; non-manual or robotic clean-up; fully automated and safer handling of infectious biological fluids, on-column enrichment of analytes, low cost per sample and last but not least improved precision, and accuracy.

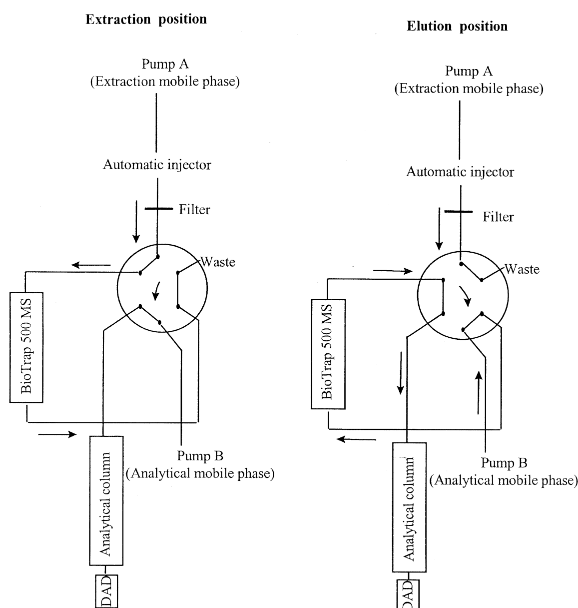


Fig. 1. Schematic representation of the column-switching set-up. Adapted from Ref. [14].

2. Experimental

2.1. Chemicals

Clonazepam, diazepam, flunitrazepam, midazolam and oxazepam were purchased from Promochem (Molsheim, France). Human plasma was obtained from the University Hospital of Geneva (Switzerland).

Monobasic and dibasic potassium phosphate, phosphoric acid were purchased from Merck (Darmstadt, Germany), acetonitrile (HPLC-grade) was obtained from Romil (Cambridge, UK).

2.2. Instrumentation

A schematic representative column-switching HPLC technique set-up is given in Fig. 1 [14]. The on-line system consisted of two quaternary pumps (Model HP 1100; Hewlett-Packard) and two columns, connected by a HP 1100 high-pressure six-port valve in back-flush configuration. The chromatographic system is also equipped with a diode-array detector, an automatic injector and an autosampler.

In the **extraction position**; pump A is pumping the extraction mobile phase through the autosampler, where the sample is injected. After the autosampler a filterholder is inserted with a 2 μm biocompatibility filter. After the passage through the filter, the sample is transported to the extraction column via the six-port valve. During this time, the pump B (analytical pump) is pumping the analytical mobile phase through the analytical column via the six-port valve.

In the **elution position**; the mobile phase from the pump A (the extraction mobile phase) is going to waste. And the mobile phase from pump B (the analytical mobile phase) is back-flushing from the extraction column to the analytical column.

The absorbance of the eluent was monitored at 254 nm. An HP Chemstation (Hewlett-Packard Software G2170AA) was used for instrument control, data acquisition and data handling.

2.3. Columns and mobile phases

The column-switching procedure was achieved by using a BioTrap 500 MS (Chromtec, Hägersten, Sweden) a new biocompatible extraction column, offering repeated direct injection of serum, plasma, supernatant of cell culture or other complex matrices, into the HPLC system without any clean-up procedure (except a simple centrifugation). This biocompatible extraction column is pH stable (pH 2 to 11)

with a biocompatible external surface and a hydrophobic internal surface (hydrophobic polymer).

The surface within the pores is also a hydrophobic polymer and the pores of matrix are small enough to exclude the plasma protein and other macromolecular compounds.

The separation was performed using a semi micro column (C_8 reversed-phase: LiChrospher Select B, 125×3 mm I.D., 5 μm particle size) and a guard column (Nucleosil NH_2, 8×4 mm I.D., 5 μm particle size) (Macherey-Nagel, Switzerland) [13].

The column-switching mobile phase consisted of 30 m*M* phosphate buffer (pH 7.2)–acetonitrile (94:6, v/v). The analytical mobile phase was composed of a mixture of a 20 m*M* phosphate buffer (pH 2.1) and acetonitrile. The optimized conditions of the whole procedure are given in Table 1.

2.4. Sample preparation

2.4.1. Standard solutions

Stock standard solutions of clonazepam, diazepam, flunitrazepam, midazolam and oxazepam were prepared by dissolution of each compound in methanol to obtain a concentration of 1 mg/ml. These were stored at −20°C and remained stable for at least 24 months.

Biological standards were prepared at concentrations of 50, 250, 500, 1000, 2000, 3000 and 5000 ng/ml for both oxazepam and diazepam, and at concentrations of 50, 100, 300, 500, 800 and 1000 ng/ml for clonazepam; flunitrazepam and midazolam, by diluting the appropriate aliquots of the stock solutions with drug-free plasma.

2.4.2. Phosphate buffer

The extraction phosphate buffer (pH 7.5, 30 m*M*) was prepared by transferring 2.72 ml of 1 *M* KH_2PO_4 and 9.9 ml of 1 *M* K_2HPO_4 to a 1000-ml volumetric flask, and made up with distilled water to the volume. The analytical phosphate buffer (pH 2.1, 20 m*M*) was prepared by transferring 12.72 ml of 1 *M* KH_2PO_4 and 22.33 ml of 1 *M* H_3PO_4 to a 1000-ml volumetric flask, and made up with distilled water to the volume. Buffer solutions are always freshly prepared and filtered through a 0.45-μm filter (Supelco, Bellefonte, PA, USA) immediately before use.

2.4.3. Plasma samples

Untreated plasma samples (1 ml) were spiked with the mentioned benzodiazepines at the desired concentrations and with 20 μl of the appropriate internal standard (methylclonazepam, 100 μg/ml). After centrifugation the spiked samples were placed to

Table 1
Optimized conditions of the whole procedure

Extraction column	Biotrap MS 500, 20×4.0 mm I.D. Thermostated at 35°C
Analytical column	LiChrospher Select B, 125×3 mm I.D., 5 μm Thermostated at 35°C
Loading	K_2HPO_4 (30 m*M*, pH 7.2) 5 min at 0.6 ml/min
Transfer and analytical separation	A=Acetonitrile (ACN) B=KH_2PO_4 (20 m*M*, pH 2.1) Linear gradient 0 min A–B (30:70) at 0.5 ml/min 5 min: A–B (30:70) at 0.5 ml/min 30 min: A–B (35:65) at 0.3 ml/min
Detection	254 nm
Volume of plasma injected	50 μl

glass vials and 50 μl were processed on-line as described above.

3. Method development

The automated method proposed was optimized before validation. We studied the influence of experimental extraction conditions on the elution of BZDs by varying the following parameters of extraction mobile phase: acetonitrile (ACN) percent; buffer pH; buffer concentration; temperature and flow-rate.

3.1. Acetonitrile percent

We studied the influence of varying ACN in the extraction mobile phase from 0 to 6%. Although the peak area was altered by varying this parameter, increasing the percent of organic solvent had a positive influence on the peak area (Fig. 2).

Preliminary tests done in order to study the compatibility between the BioTrap 500 MS and the extraction mobile phase showed that with more than 8% of ACN in the mobile phase plasma protein precipitation could be observed in the column.

3.2. Buffer pH

We studied the effect of varying, between 6.0 and 7.5, the pH of the extraction mobile phase. Varying this parameter did not significantly alter the peak area (Fig. 2).

3.3. Buffer concentration

We examined the effect of varying buffer concentration from 20 to 40 m*M*. Despite varying the buffer concentration the peak area was not significantly altered, the best chromatogram was obtained at a 30 m*M* buffer concentration compared to the chromatogram obtained at 10, 20 and 40 m*M* (Fig. 3a and b).

3.4. Temperature

Varying the temperature from 10 to 60°C, altered the peak area. We observed an increase in the peak area, following by a stabilisation between 35 and 45°C, then decreasing values at 50°C (Fig. 2). Therefore an optimum temperature of 35°C was chosen in order to avoid any damage to the external surface (α_1-acid glycoprotein) by high temperature.

3.5. Flow-rate

The study of variation of the flow-rate from 0.6 to 2.0 ml/min, showed that the peak area decreased with increasing flow-rate. Fig. 2 showed a similar decrease in peak area for all the compounds. The explanation for this decrease is that at high flow-rates the compounds did not have time to penetrate the pores of particles and be retained by the hydrophobic inner surface of the particles before being eluted by the analytical mobile phase.

4. Results

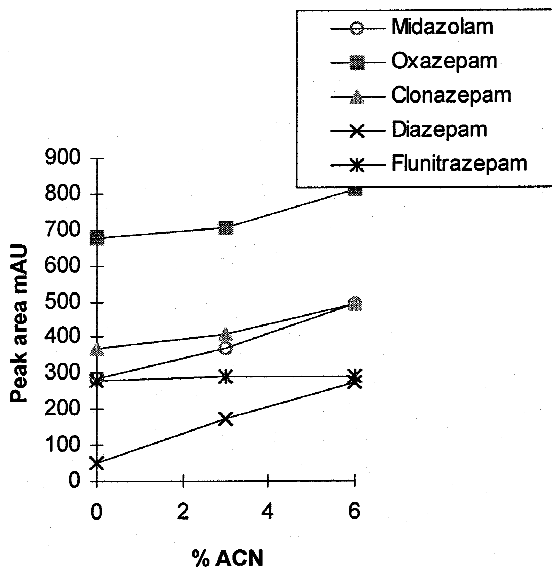
4.1. Linearity

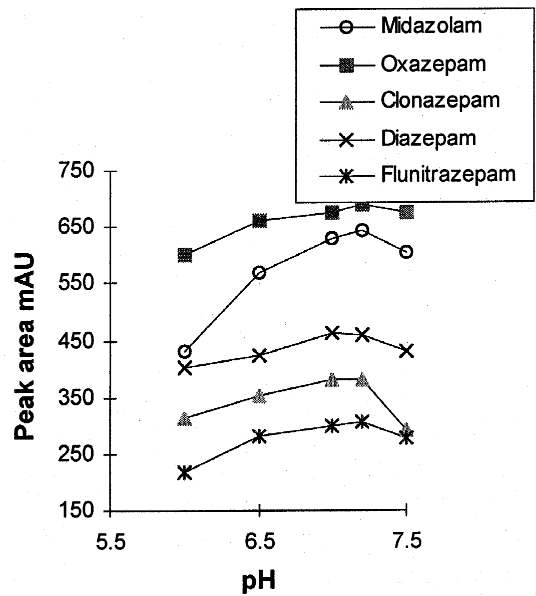
Detector response linearity studies were performed by preparing five triplicate calibrations covering the range of following concentrations: 50 to 1000 ng/ml for clonazepam, flunitrazepam, midazolam and 50 to 5000 ng/ml for diazepam and oxazepam. Linear regression lines were obtained by plotting the peak area ratios (the compound peak area divided by one of the internal standard, see Table 2).

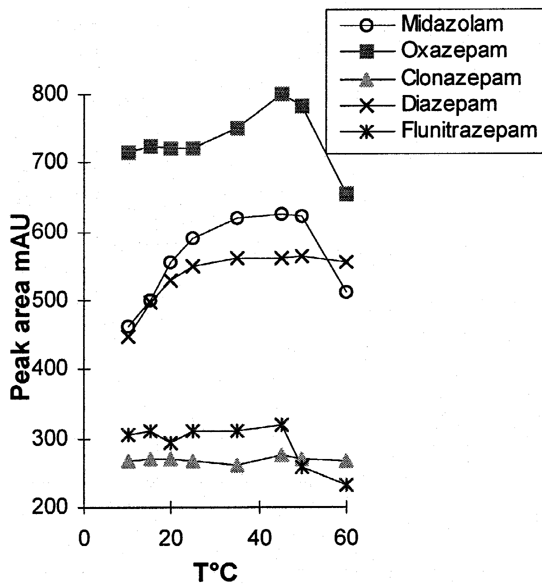
4.2. Precision

Method precision was determined by measuring repeatability (intra-day precision) and reproducibility (between-day precision) of peak areas for all BZDs studied.

In order to evaluate the repeatability of the method, replicate injection ($n=6$) of three different concentrations: 250, 3000 and 5000 ng/ml for diazepam and oxazepam and 100, 300 and 500 ng/







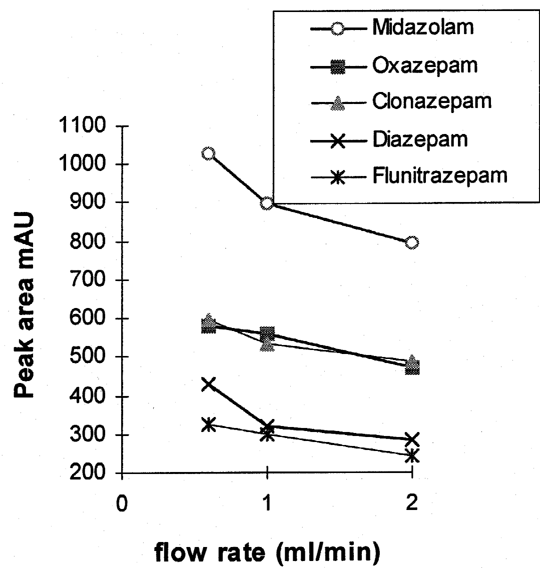


Fig. 2. Effect of experimental parameters on the peak area for the different benzodiazepines.

ml for the other three with 2000 ng/ml of internal standard were carried out. As shown in Table 3, relative standard deviation (RSD) values are given for the five benzodiazepines mentioned.

The reproducibility was also evaluated over 3 days by performing daily six replicate injections. Results (see Table 3) show that the RSD values were satisfactory for all the BZDs studied.

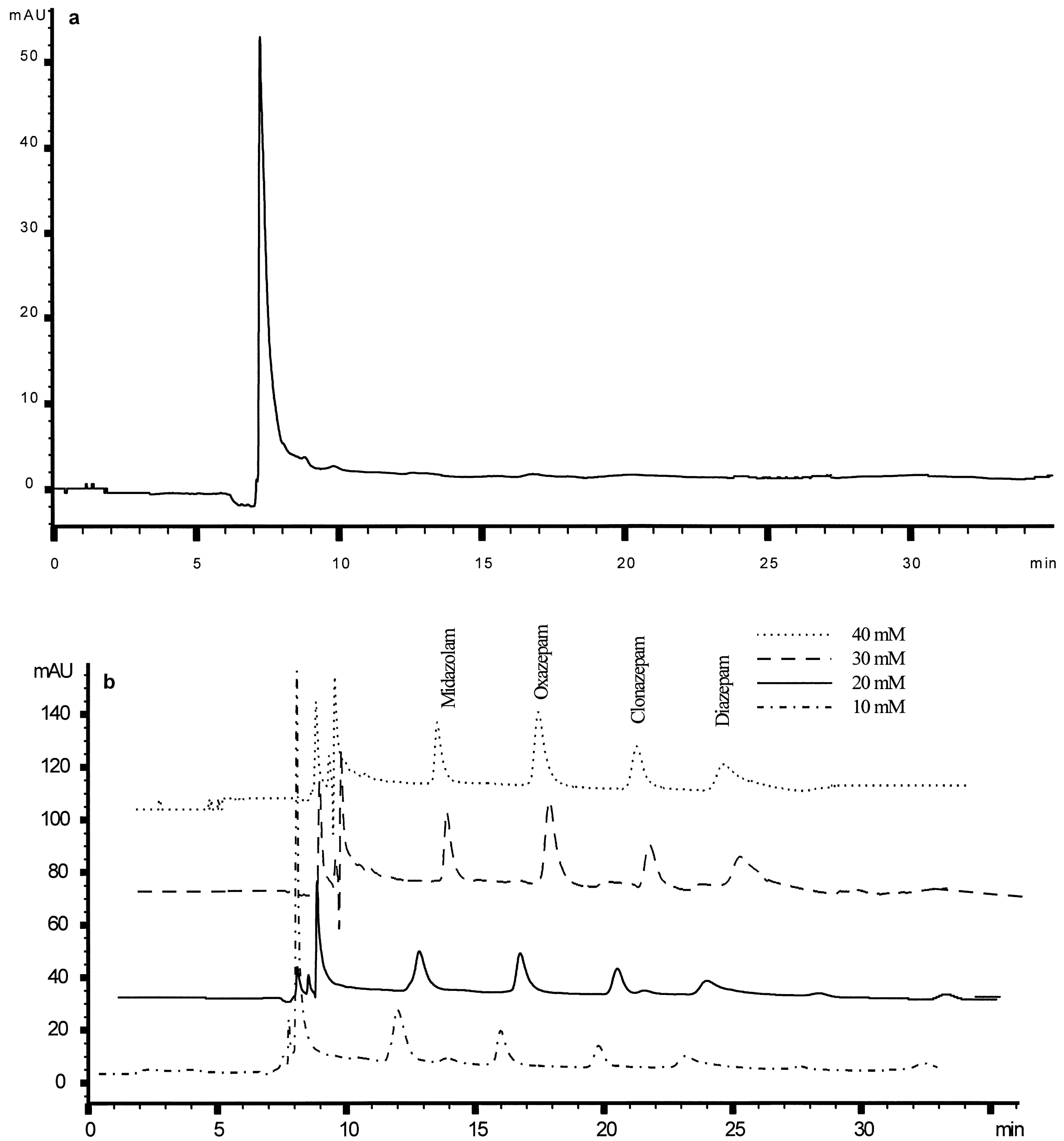


Fig. 3. (a) A chromatogram trace of blank plasma. (b) Effect of buffer concentration on the chromatogram trace.

4.3. Assay detection limits

4.3.1. Limit of detection (LOD)

The LOD, defined as the lowest concentration of the analyte that can be clearly detected above the baseline signal, is estimated as three-times the signal-to-noise ratio. The LOD was determined ($n=6$) by injection of a spiked plasma with BZDs in decreasing concentrations. The LOD was determined around 15 ng/ml for clonazepam, flunitrazepam and

Table 2
Calibration data for the five benzodiazepines ($n=3$)

Compound	Range (μg/ml)	Correlation coefficient (r)	Regression equation
Clonazepam	0.05–1.0	0.997	$0.14\cdot10^{-2}x+0.71\cdot10^{-2}$
Diazepam	0.05–5.0	0.997	$0.18\cdot10^{-2}x-0.49\cdot10^{-1}$
Flunitrazepam	0.05–1.0	0.997	$0.2\cdot10^{-2}x-0.42\cdot10^{-1}$
Midazolam	0.05–1.0	0.997	$0.13\cdot10^{-2}x+0.12\cdot10^{-2}$
Oxazepam	0.05–5.0	0.998	$0.2\cdot10^{-2}x-0.23$

Table 3
Repeatability and reproducibility ($n=6$)

Concentration (ng/ml)	Clonazepam		Flunitrazepam		Midazolam	
	Repeatability (RSD, %)	Reproducibility (RSD, %)	Repeatability (RSD, %)	Reproducibility (RSD, %)	Repeatability (RSD, %)	Reproducibility (RSD, %)
50	14.2	15.9	15.7	20.2	14.21	8.2
300	3.5	3.7	2.7	3.5	4.1	3.7
500	0.9	1.5	1.1	2.3	3.1	2.4
	Oxazepam		Diazepam			
	Repeatability (RSD, %)	Reproducibility (RSD, %)	Repeatability (RSD, %)	Reproducibility (RSD, %)		
250	7.5	10.0	10.1	9.2		
3000	2.7	3.8	2.6	2.8		
5000	2.1	2.6	1.00	2.3		

Table 4
Recoveries obtained with spiked plasma samples ($n=6$)

Amount added (ng/ml)	Mean recovery (%)		
	Clonazepam	Flunitrazepam	Midazolam
50	91.0	82.0	106.0
100	98.3	98.0	99.0
300	102.0	97.3	108.0
500	101.0	112.0	105.0
800	104.0	110.0	103.7
1000	100.7	104.0	101.0
	Oxazepam	Diazepam	
250	109.0	109.0	
500	125.0	109.0	
1000	111.7	103.0	
2000	105.3	99.3	
3000	103.6	103.7	
5000	109.1	108.7	

midazolam, 18 ng/ml for oxazepam and 24 ng/ml for diazepam.

4.3.2. Limit of quantification (LOQ)

The LOQ was determined ($n=6$) by injection of a spiked plasma with BZDs in decreasing concentrations. The reproducibility was calculated for each concentrations. Then, LOQ was calculated as the concentration where the reproducibility was below 15% (Fig. 4). LOQ was determined as about 50 ng/ml for clonazepam, flunitrazepam, midazolam and 80 ng/ml for diazepam and 60 ng/ml for oxazepam.

4.4. Recovery

The absolute recoveries of the five BZDs studied were determined by comparing the peak areas of spiked plasma samples and reference samples at six

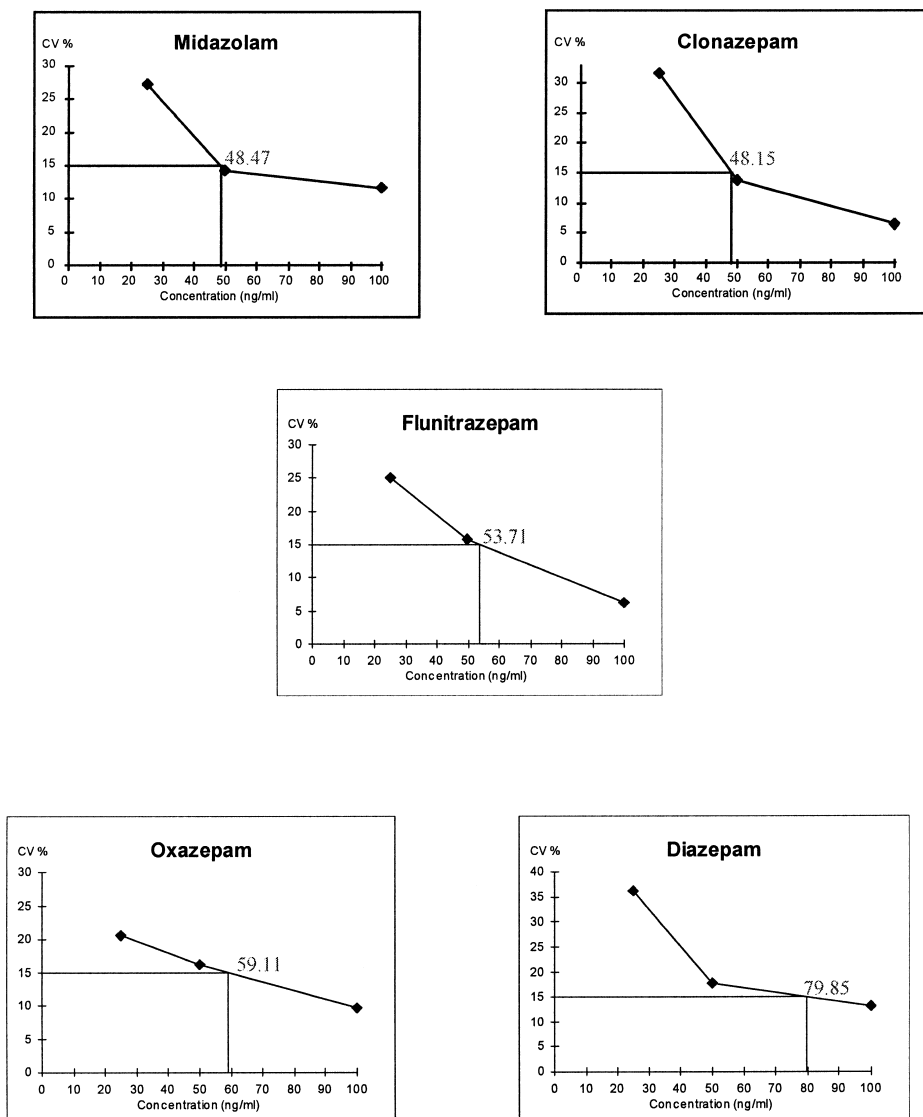


Fig. 4. Curve determination of LOQ values.

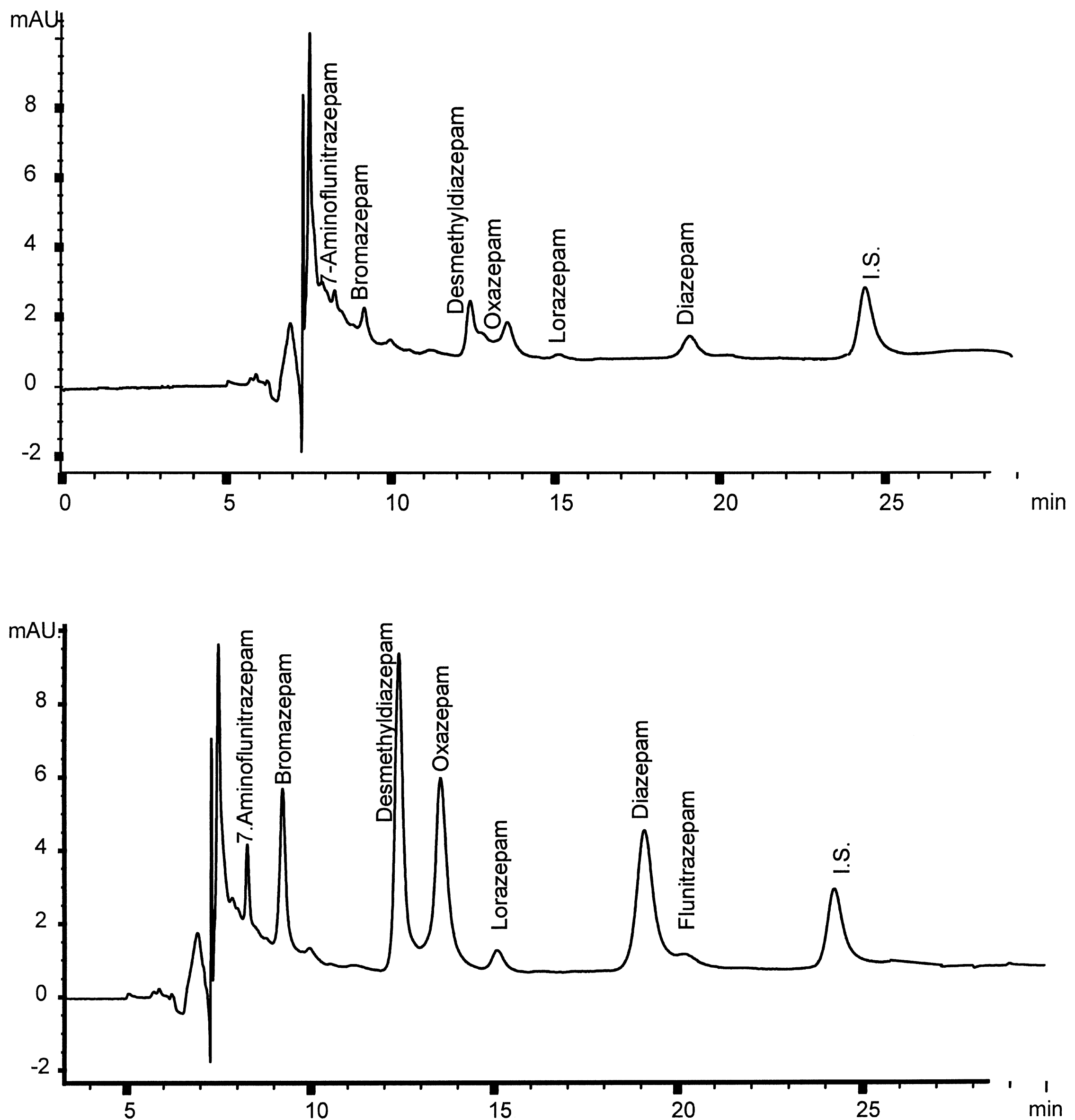


Fig. 5. Chromatograms of the certified serum at level 1 (top) and at level 2 (bottom).

different concentrations. The reference samples were injected directly onto the analytical column and the spiked plasma samples were injected onto the extraction column coupled to the analytical column through the switching valves.

As shown in Table 4, the method developed gives excellent recovery values.

We observed that the recovery is better than 98% for all the BZDs except for flunitrazepam and clonazepam at low concentrations.

4.5. Accuracy

The accuracy of the whole procedure was verified with a certified standard serum provided by Medichem (Steinbronn, Germany). This serum contained

Table 5
Accuracy determined with a certified standard serum at two levels

Compound	Retention time (min)	Level 1			Level 2		
		Concentration measured (ng/ml)	Concentration certified (ng/ml)	Difference (%)	Concentration measured (ng/ml)	Concentration certified (ng/ml)	Difference (%)
7-Amino-flunitrazepam	8.27	n.q.	10	–	49.0	50	−2.0
Bromazepam	9.18	92.0	100	−7.0	439.0	400	+9.8
Diazepam	19.09	103.0	100	+3.0	629.0	600	+4.8
Flunitrazepam	20.19	n.q.	10	–	56.0	50	+12.0
Lorazepam	15.11	16.8	20	−17.5	83.0	100	−17.0
Nordiazepam	12.40	99.0	100	−1.0	652.0	600	+8.7
Oxazepam	13.54	99.0	100	−1.0	640.0	600	+6.7

n.q.=Not quantified, below the LOQ.

three previously studied benzodiazepines: diazepam, flunitrazepam and oxazepam; two other benzodiazepines: bromazepam, lorazepam; and finally two important metabolites: 7-aminoflunitrazepam and desmethyldiazepam. The results presented in Table 5, were in good agreement with the certified values. Under these conditions the BZDs are well separated (see Fig. 5).

5. Conclusion

The whole HPLC procedure described here for simultaneous determination of benzodiazepines appears rapid and suitable for routine analysis.

Satisfactory validation data were achieved for linearity, precision and recovery. The LOQ obtained allowed one to measure therapeutic concentrations for most of the benzodiazepines and toxic concentrations for flunitrazepam.

The accuracy of this method was verified with reference standard serum and excellent agreement with the certified values was obtained. The use of a column-switching technique allows for safer handling of infectious biological fluids like blood and plasma.

Acknowledgements

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References

[1] K. Jinno, M. Taniguchi, M. Hayashido, J. Pharm. Biomed. Anal. 17 (1998) 1081–1091.
[2] E. Tanaka, M. Terada, S. Misawa, C. Wakasugi, J. Chromatogr. B 682 (1996) 173–178.
[3] J.B. Roberts, J.A. Tafuri, in: L.M. Haddad, J.F. Winchester (Eds.), Clinical Management of Poisoning and Drug Overdose, W.B. Saunders, Philadelphia, PA, 1990, pp. 800–820.
[4] A.G. Verstaete, F.M. Belpaire, G.G. Leroux-Roels, J. Anal. Toxicol. 22 (1998) 27–32.
[5] C.M. Moore, K. Sato, Y. Katsumata, J. Clin. Chem. 37 (1991) 804–808.
[6] K. Kudo, T. Nagata, K. Kimura, T. Imamura, M. Noda, J. Chromatogr. 431 (1988) 351–359.
[7] N. De Giovanni, M. Chiarotti, J. Chromatogr. 428 (1988) 321–329.
[8] H. Maurer, K. Pfleger, J. Chromatogr. 422 (1987) 85–101.
[9] M. Japp, K. Garthwaite, A.V. Geeson, M.D. Osselton, J. Chromatogr. 439 (1988) 317–339.
[10] A.J.H. Louter, E. Bosma, J.C.A. Schipperon, J.J. Vreuls, U.A.Th. Brinkman, J. Chromatogr. B 689 (1997) 35–43.
[11] D. Westerlund, Chromatographia 24 (1987) 155–164.
[12] J.B. Lecaillon, N. Febvre, C. Souppart, J. Chromatogr. 317 (1984) 493–506.
[13] A. El Mahjoub, C. Staub, J. Pharm. Biomed. Anal., in press.
[14] J. Hermansson, A. Grahn, J. Chromatogr. 660 (1994) 119–129.