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Simultaneous determination of benzodiazepines in whole blood or serum by HPLC/DAD with a semi-micro column

Anissa El Mahjoub, Christian Staub *

Institut Universitaire de Médecine Légale, 9, Avenue de Champel, 1211 Geneva 4, Switzerland

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Abstract

A simple and sensitive high performance liquid chromatographic (HPLC) method using a semi-micro column, C_8 reversed-phase column (3 mm i.d.) and a low flow rate 0.3 ml/min was developed and validated for the determination of five frequently prescribed benzodiazepines: clonazepam, diazepam, flunitrazepam, midazolam and oxazepam. Quantification was performed at 220 nm with methylclonazepam as internal standard. The method involved a simple extraction from alkalinized blood (1 ml) into 1-chlorobutane and provided excellent sensitivity, recovery, accuracy and reproducibility for benzodiazepines in therapeutic or toxic concentrations. © 2000 Elsevier Science B.V. All rights reserved.

Keywords: Benzodiazepines; Blood; Diode array detection; High performance liquid chromatography; Serum; Semi-micro HPLC column

1. Introduction

Benzodiazepine (BZD) drugs are widely prescribed for their anxiolytic, hypnotic, anticonvulsive and muscle relaxing properties; they are important in treating a variety of medical disorders but are also subject to abuse [1–3]. Extraction and identification of BZD in human fluids is thus very important for forensic and clinical toxicology and laboratories are frequently requested to determine BZD in serum, whole blood, urine, or gastric fluid.

However, analysis of BZD in body fluids is complex because of the diversity of BZD available on the market and the fact that each drug has a particular therapeutic and toxic range [4].

A variety of methods described in the literature allow the detection and determination of BZD in biological matrices.

Gas chromatography/mass spectrometry (GC/MS) methods have been frequently reported [5–9]. Further studies have been carried out [17–19], and a comparison between this GC/MS technique and various immunoassays have been made [25,26].

But, GC is not suitable for thermally unstable drugs and needs a supplementary derivatization step in order to improve compound volatility.

* Corresponding author. Tel.: +41-22-702-5608; fax: +41-22-789-2417.
E-mail address: christian.staub@medecine.unige.ch (C. Staub).

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Therefore, several high performance liquid chromatographic (HPLC) methods have been reported for the determination of BZD and their major metabolites [8,10–13].

For example, gradient elution and diode array detection (DAD), have been published [14,15].

More recently, Drummer [16] published a paper reviewing over 5 years the methods dedicated to measuring BZD in biological samples.

The present paper focuses on the use of an HPLC procedure based on a fast isocratic elution and a semi-micro column (3 mm i.d.) for a good separation in the simultaneous determination of several benzodiazepines. A semi-micro column needs less solvent for the mobile phase than a conventional HPLC column.

The eluent is monitored by DAD and allows both identification and quantification of benzodiazepines.

The proposed method was tested with the five following benzodiazepines: clonazepam, diazepam, flunitrazepam, midazolam and oxazepam. The structures of these compounds [20] are given in Fig. 1.

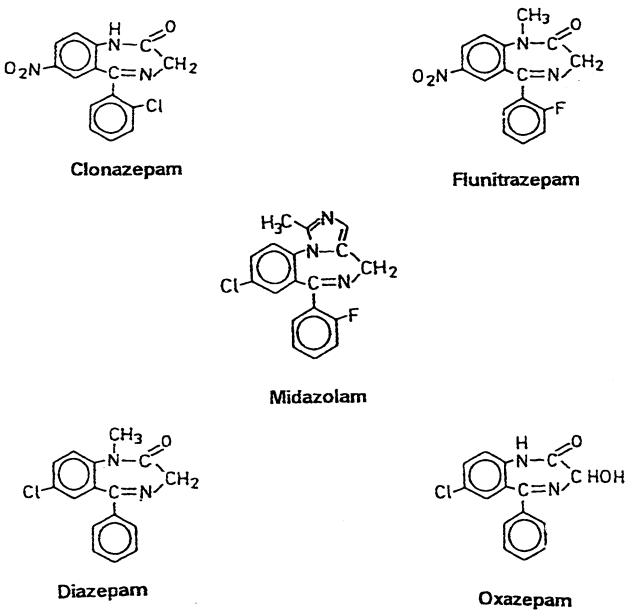

Fig. 1. Structures of the five main benzodiazepines studied: clonazepam, diazepam, flunitrazepam, midazolam and oxazepam.

2. Experimental

2.1. Chemicals

Clonazepam, diazepam, flunitrazepam, midazolam and oxazepam were purchased from Promochem (Molsheim, France). Human blood was obtained from the University Hospital of Geneva (Switzerland). Monobasic potassium phosphate and phosphoric acid were purchased from Merck (Darmstadt, Germany) and acetonitrile HPLC grade was provided by Romil (Cambridge, UK).

2.2. Instrumentation and chromatographic procedure

An HPLC model 1100 (Hewlett–Packard, Palo Alto, CA, USA), was selected with a quaternary pump equipped with a diode-array detector, an automatic injector and an autosampler. The separation was achieved using a C_8 reversed-phase column: Lichrospher Select B 125 × 3 mm i.d. with 5 μm particle size and a guard column Nucleosil NH_2 8 × 4 mm i.d. with 5 μm particle size (Macherey–Nagel, Switzerland).

The mobile phase was a mixture of phosphate buffer (20 mM, pH 2.1) and acetonitrile (65:35 v/v). The buffer solution was filtered through a 0.45-μm filter (Supelco, Bellefonte, PA, USA) before use.

The flow rate was 0.3 ml/min and the absorbance of the eluent was monitored at 220 nm. The column was thermostated at 25°C.

Three injections of a standard solution consisting of flunitrazepam and its metabolites, 7-aminoflunitrazepam, 7-acetamidoflunitrazepam and desmethylflunitrazepam, in presence of the internal standard, were performed prior to each sequence in order to verify the performances of the system. A Chemstation software G2170AA installed on a PC Vectra (model Vl4, Hewlett–Packard) was used for instrument control, data acquisition and data handling.

2.3. Sample preparation

2.3.1. Standard solutions

Stock standard solutions were prepared by dissolving each benzodiazepine in methanol to ob-

tain a concentration of 1 mg/ml. These solutions were stored at −20°C and remained stable for at least 12 months.

Blood standard samples were prepared by dilution of the stock solutions with drug free blood at the following concentrations: 250, 500, 1000, 2000, 3000 and 5000 ng/ml for both oxazepam and diazepam; at 30, 50, 100, 200, 300 and 500 ng/ml for clonazepam and midazolam and finally at 10, 50, 100, 200, 300 and 500 ng/ml for flunitrazepam.

2.3.2. Phosphate buffer

The phosphate buffer was prepared by transferring 12.72 ml of 1 M KH_2PO_4 and 22.33 ml of 1 M H_3PO_4 into a 1000-ml volumetric flask, and made up to volume with distilled water. Buffer solution was always freshly prepared and filtered immediately before use.

2.3.3. Extraction

A total of 1.0 ml of blood, 30 μl of the appropriate internal standard (methylclonazepam, 10 μg/ml) and 50 μl of ammonia solution 25% were added to a glass tube with a Teflon lined screw cap.

The solution was briefly mixed, and 5 ml of 1-chlorobutane (n-butyl chloride) was added. The tube was capped tightly. After vertical agitation for 2 min and centrifugation at 5000 rpm for 10 min, the upper organic phase was transferred to a clean conical tube and evaporated under a gentle stream of nitrogen. The residue was reconstituted by adding 50 μl of the mobile phase. A total of 20 μl was injected into the chromatographic system.

3. Results

3.1. Chromatography

Under the described chromatographic conditions, the five BZD were well separated. However, the method was validated in two steps, first for oxazepam and diazepam, and then for the three other BZD. Fig. 2 shows chromatograms of drug free blood and of blood spiked with 150–400 ng/ml of each compound.

3.2. Linearity

Detector response linearity was performed by preparing five triplicate calibration samples covering the range between therapeutic and toxic concentrations.

Calibration curves (Table 1) were obtained from spiked blood samples at the following concentration ranges: 30–500 ng/ml for clonazepam and midazolam; 250–5000 ng/ml for diazepam and oxazepam; and finally 10–500 ng/ml for flunitrazepam.

3.3. Precision

The intraday precision or repeatability (showed in Table 2) was evaluated by replicate analysis ($n = 6$) of pooled blood at three different concentrations: 250, 3000, and 5000 ng/ml for diazepam and oxazepam; 30, 300 and 500 ng/ml for the three others.

The interday precision or reproducibility (showed in Table 3) was also evaluated by replicate analysis ($n = 6$) over 3 days, of the same blood samples used to determine repeatability.

3.4. Assay detection limits

3.4.1. Limits of detection (LOD)

The limit of detection (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. LOD was determined ($n = 6$) after extraction of spiked blood, by injection, with BZD in decreasing concentrations. LOD was determined as 3.5 ng/ml for clonazepam, flunitrazepam and midazolam, and 2 ng/ml for diazepam and oxazepam.

3.4.2. Limit of quantification (LOQ)

The LOQ was obtained by the same procedure used for LOD, but estimated as ten times the signal to noise ratio [27]. LOQ values were determined as 10 ng/ml for clonazepam, flunitrazepam and midazolam, and 5 ng/ml for diazepam and oxazepam. LOQ values were subsequently validated by the analysis of six samples known to be at the limit of quantification [28].

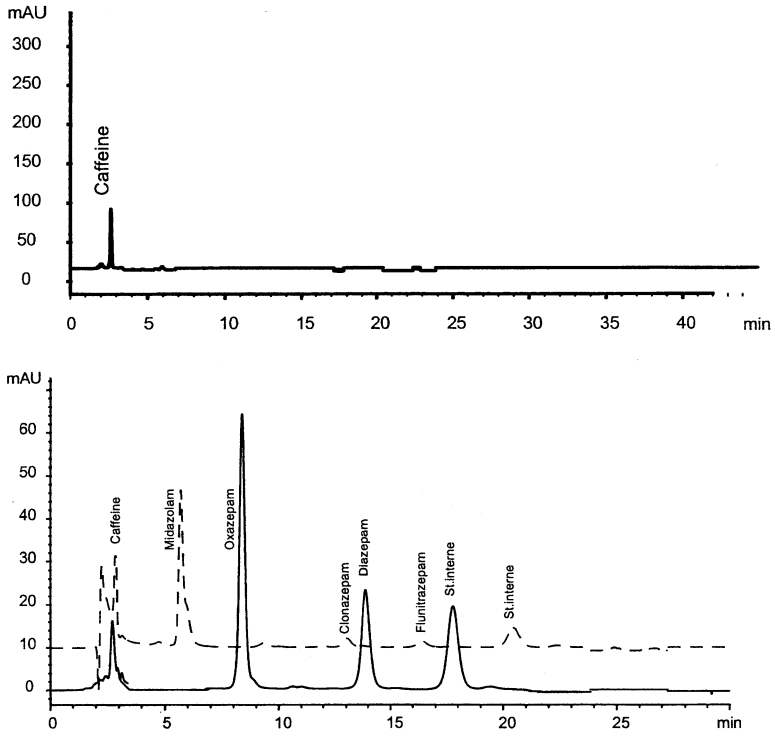

Fig. 2. Chromatograms of a drug free blood sample (top) and a spiked blood with clonazepam (150 ng/ml), flunitrazepam (150 ng/ml), midazolam (400 ng/ml), diazepam (250 ng/ml), and oxazepam (250 ng/ml), and methylclonazepam (300 ng/ml I.S.). Analytical conditions: injection, 20 μl of extracted blood on C_8 reversed-phase column: Lichrospher Select B (125 × 3 mm i.d.), mobile phase, acetonitrile-phosphate buffer (pH 2.1; 0.2 M) (35:65 v/v) at a flow rate of 0.3 ml/min UV detection at 220 nm.

Table 1
Calibration data for the five benzodiazepines ($n = 6$)

	Range (μg/ml)	Coefficient of correlation r	Slope	Intercept
Clonazepam	0.03–0.5	0.998	0.007	0.043
Diazepam	0.25–5.0	0.999	3.504	−0.083
Flunitrazepam	0.01–0.5	0.998	0.007	0.061
Midazolam	0.03–0.5	0.996	0.006	0.023
Oxazepam	0.25–5.0	0.998	3.365	−0.018

The coefficient of variation ($n = 6$) at these LOQ values was 2% for oxazepam, 6% for clonazepam and diazepam, 15% for flunitrazepam and midazolam.

3.5. Recovery

The efficiency of liquid–liquid extraction was measured by spiking drug-free blood with known concentrations of standards. Six extraction solvents were investigated which included chloroform [2], ethylacetate–hexane 30:70 [16], toluene [21], cyclohexane–dichloromethane 40:60, ethylacetate [22], and toluene–hexane–isoamylalcohol 78:20:2 [23, 24]. 1-Chlorobutane was chosen as the extraction solvent because it provides the best recovery (> 90%) for all the BZD studied (Table 3), except for oxazepam for which the recovery was about 60%.

Table 2
Repeatability and reproducibility ($n = 6$)

Concentration (ng/ml)	Clonazepam		Flunitrazepam		Midazolam	
	Intraday precision CV%	Interday precision CV%	Intraday precision CV%	Interday precision CV%	Intraday precision CV%	Interday precision CV%
30	10.9	13.8	4.7	4.9	5.8	9.7
300	7.2	7.9	3.6	4.3	4.9	5.3
500	1.8	4.7	2.5	3.5	2.0	4.9
	Oxazepam		Diazepam			
	Intraday precision CV%	Interday precision CV%	Intraday precision CV%	Interday precision CV%		
250	3.5	4.6	3.5	5.6		
3000	3.5	4.1	3.4	4.4		
10 000	3.6	4.1	3.4	4.2		

3.6. Accuracy

The accuracy of the whole procedure was verified with a certified standard serum provided by Medichem (Steinenbronn, Germany). This serum contained three benzodiazepines previously studied: diazepam, flunitrazepam and oxazepam, two other benzodiazepines: bromazepam and lorazepam, and finally two important metabolites: 7-aminoflunitrazepam and desmethyldiazepam. The results presented in Table 4 were in good agreement with the certified values.

The use of DAD detection allows a reliable identification of benzodiazepines even at low concentrations (Fig. 3).

In order to better separate diazepam from flunitrazepam, the following gradient elution program was applied: 30–35% of acetronitrile in 25 min. The other chromatographic parameters remained unchanged.

3.7. Ruggedness

The method ruggedness (Fig. 4) was tested by varying several chromatographic parameters and studying the effect on column efficiency (represented by the number of theoretical plates N).

3.7.1. Mobile phase pH

Varying the mobile phase pH between 2.0 and 3.0 did not significantly alter column efficiency.

Furthermore, working at pH 2.1 did not lead to any decrease in column performance. Under these conditions, more than 850 injections were achieved.

3.7.2. Mobile phase composition

Varying the acetonitrile percent from 30 to 38% did not significantly alter column efficiency.

3.7.3. Flow rate

Variation of the flow rate from 0.2 to 0.6 ml/min, showed that column efficiency decreased when the flow rate increased. However, 0.3 ml/min seems to be a good compromise when considering the chromatographic system and solvent economy.

At this flow rate, reproducibility of retention time ($n = 6$) was always better than 1%.

3.7.4. Temperature

Varying the temperature between 10 and 30°C significantly altered column efficiency. Therefore a controlled temperature of 25°C was chosen.

Table 3
Recoveries obtained with spiked blood samples ($n = 6$)

Amount added ng/ml	Clonazepam (mean recovery %)	Flunitrazepam (mean recovery %)	Midazolam (mean recovery %)
30	93.0	82.1	85.2
50	92.5	87.4	98.6
100	78.7	86.7	94.0
200	80.7	n.d.[a]	91.4
300	84.7	89.9	94.0
500	87.5	99.3	108.9
	Oxazepam (mean recovery %)	Diazepam (mean recovery %)	
250	60.5	96.4	
500	50.5	82.2	
1000	50.3	85.0	
2000	56.7	92.4	
3000	56.3	92.4	
5000	58.9	91.4	
10 000	60.0	87.4	

[a] n.d., not determined.

Table 4
Accuracy determined with a certified standard serum at two levels

Compound	Level 1			Level 2		
	Concentration measured (ng/ml)	Concentration certified (ng/ml)	Difference (%)	Concentration measured (ng/ml)	Concentration certified (ng/ml)	Difference (%)
Diazepam	90	100	−10.0	613	600	2.2
Desmethyldiazepam	99.9	100	−0.1	598	600	−0.3
Flunitrazepam	9.2	10	−8.0	53.8	50	6.0
7-Aminoflunitrazepam	8.0	10	−20.0	40.2	50	−19.6
Oxazepam	106.6	100	6.6	604	600	0.8
Bromazepam	85.0	100	−15.0	400	400	0
Lorazepam	20.2	20	1.0	101.3	100	1.3

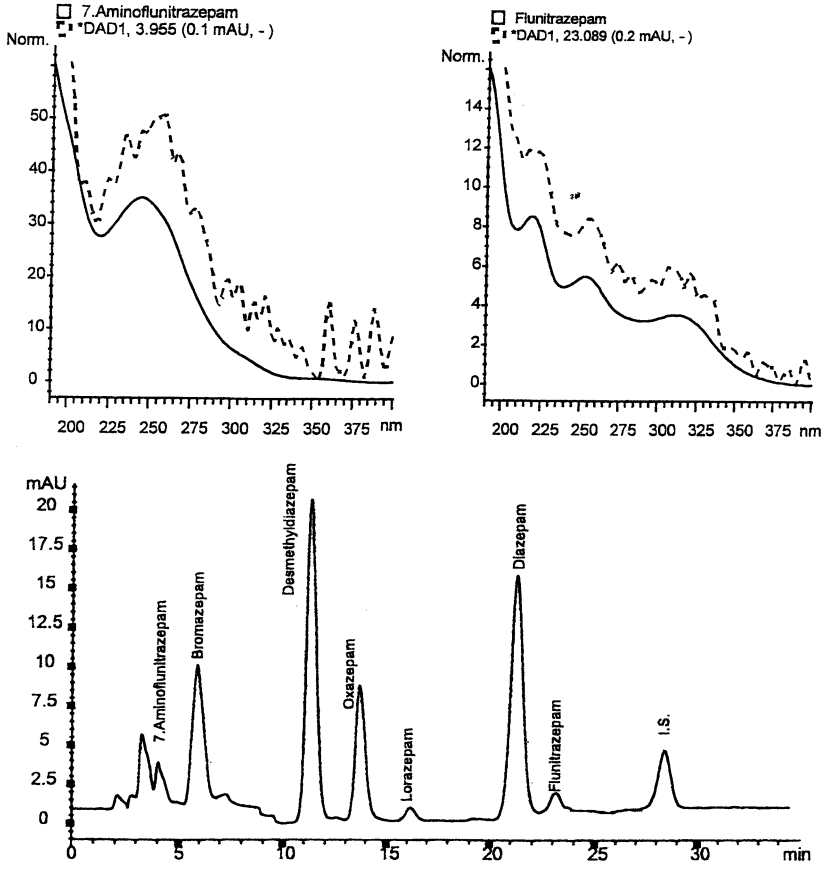

Fig. 3. Chromatograms of the certified standard serum (level 1) with the UV spectra of flunitrazepam and 7-aminoflunitrazepam at 10 ng/ml. Analytical conditions: injection, 20 μl of extracted serum on C_8 reversed-phase column: Lichrospher Select B (125 × 3 mm i.d.), mobile phase, acetonitrile-phosphate buffer (pH 2.1; 0.2 M) and a gradient elution program was applied: 30–35% of acetronitrile in 25 min at a flow rate of 0.3 ml/min UV detection at 220 nm

3.8. Application to 'post-mortem' blood samples

Generally, post-mortem whole blood is not as fluid as antemortem serum or plasma, and is not easy to work with. Therefore, several samples were chosen to demonstrate the potential of this method.

The first blood sample obtained from a deceased subject, showed two benzodiazepines (demoxepam and desmethyldiazepam), one antidepressant (amitryptiline and its main metabolite), and tramadol and propranolol were clearly identified (Fig. 5(a)). The measured concentrations were the following: demoxepam: 1060 ng/ml, desmethyldiazepam: 1380 ng/ml, amitryptiline: 630 ng/ml, nortryptiline: 1330 ng/ml.

Flunitrazepam and its main metabolites (Fig. 5(b)) were quantitated in a second blood sample obtained from another deceased subject.

The measured concentrations were the following: flunitrazepam: $<$ LOQ, 7-aminoflunitrazepam: 485 ng/ml, desmethylflunitrazepam: 30 ng/ml.

In the third blood sample obtained from a deceased subject, two benzodiazepines (desmethyldiazepam and desalkylflurazepam) with one antidepressant (venlafaxine and its metabolite) were detected (Fig. 5(c)).

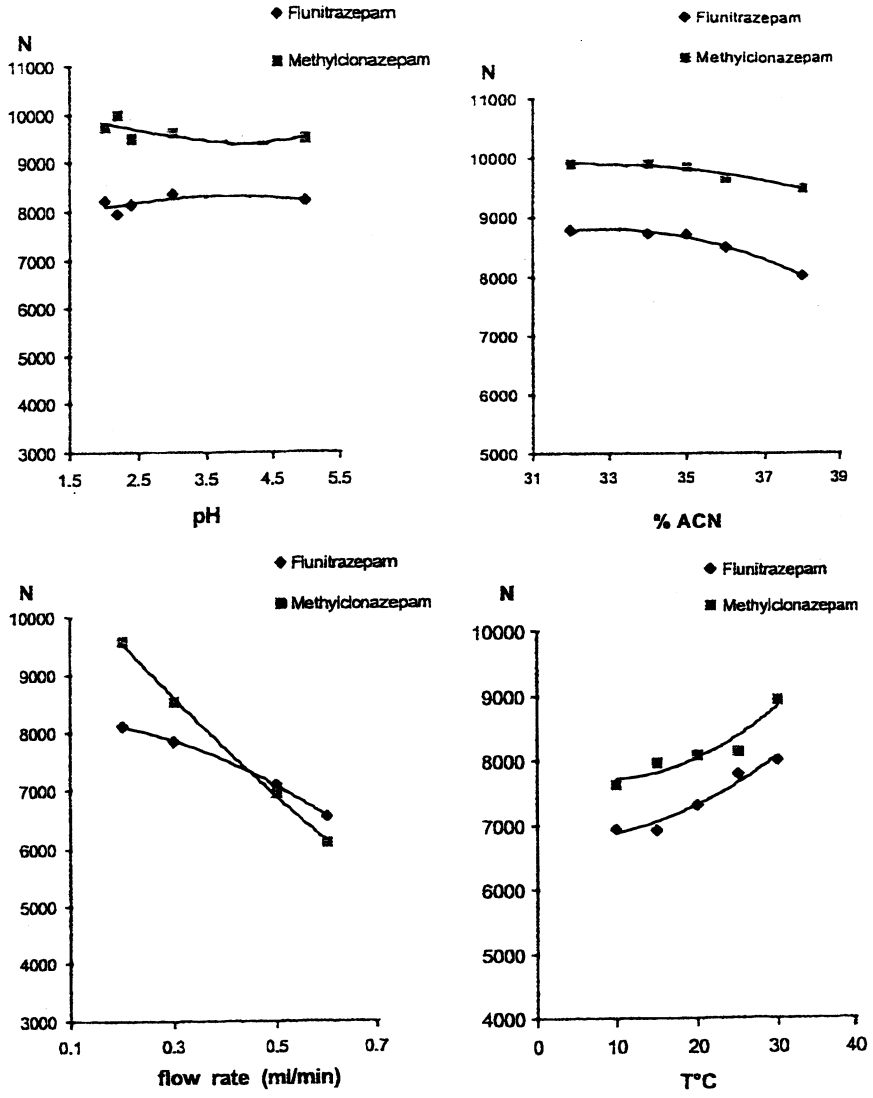

Fig. 4. Method Ruggedness: effect of the variation of several chromatographic parameters on column efficiency.

The measured concentrations were the following: diazepam: < LOQ, desmethyldiazepam: 510 ng/ml, flurazepam: < LOQ, desalkylflurazepam: 250 ng/ml, venlafaxine: 980 ng/ml desmethylvenlafaxine: 250 ng/ml.

4. Conclusion

This HPLC procedure, developed for the simultaneous determination and quantification of benzodiazepines, appears rapid, simple, and suitable for routine analysis. Satisfactory validation data were collected for linearity, precision, recovery and ruggedness, LOQ values allowed to measure therapeutic and toxic concentrations.

The accuracy of the whole procedure was verified with a reference standard serum and an acceptable to good agreement with the certified values was obtained.

The use of a semi micro column allowed to work with a low flow rate (0.3 ml/min) which

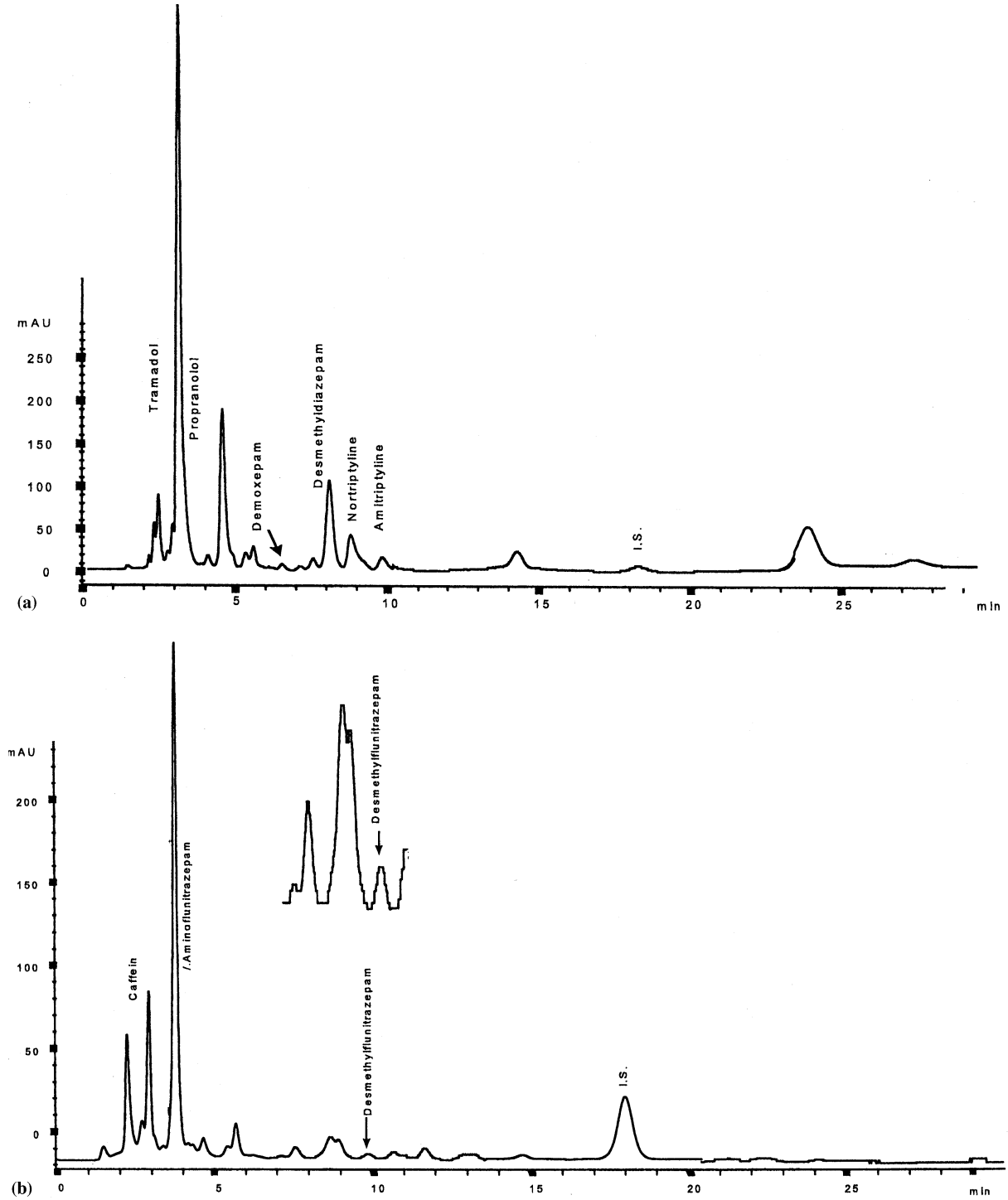

Fig. 5. Analytical conditions: injection, 20 μl of extracted blood on C_8 reversed-phase column: Lichrospher Select B (125 × 3 mm i.d.), mobile phase, acetonitrile-phosphate buffer (pH 2.1; 0.2 M) (35:65 v/v) at a flow rate of 0.3 ml/min UV detection at 220 nm. (a) Blood chromatogram of a deceased subject with demoxepam, desmethyldiazepam, amitryptiline, nortryptiline, tramadol and propranolol. (b) Blood chromatogram of a deceased subject with 7-aminoflunitrazepam and desmethylflunitrazepam. Blood chromatogram of a deceased subject with desmethyldiazepam, desalkylflurazepam, venlafaxine and desmethylvenlafaxine.

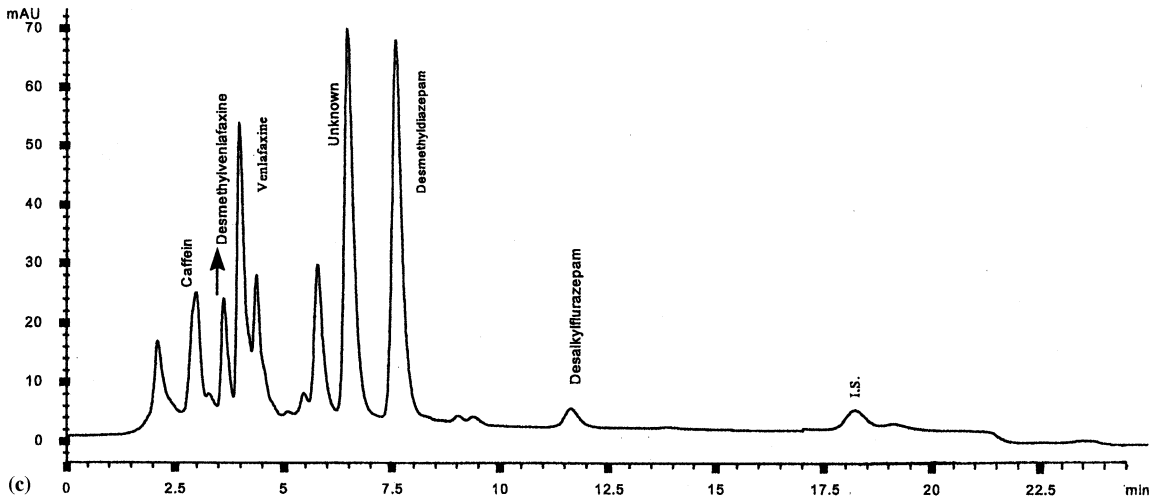

Fig. 5. (*Continued*)

meant saving solvents and contributing to environment protection. Furthermore, photodiode-array detection offered valuable assets for peak identification and peak purity determination.

With this method one can analyze 20 benzodiazepines and their metabolites in less than 20 min.

Finally, the proposed method is not only suitable for determining benzodiazepines (Table 5) in biological matrices, but could also be applied to other drugs like antidepressants (Table 6).

Table 5
Retention times (RT) and relative retention times (RRT) of benzodiazepines

Substances	RT	RRT
7-Aminoclonazepam	3.17	0.1724
7-Acetamidoflunitrazepam	3.41	0.1855
Desmethylchlordiazepoxide	3.43	0.1866
7-Aminoflunitrazepam	3.73	0.2029
Chlordiazepoxide	3.68	0.2002
Bromazepam	4.88	0.2655
Demoxepam	5.42	0.2949
Midazolam	5.46	0.2971
Clorazepate	7.88	0.4287
Desmethyldiazepam	7.91	0.4304
Oxazepam	8.52	0.4635
Desmethylfunitrazepam	10.01	0.5446
Clonazepam	11.29	0.6143
Alprazolam	12.47	0.6785
Temazepam	13.06	0.7106
Diazepam	14.30	0.7782
Triazolam	14.34	0.7802
Lormetazepam	14.59	0.7938
Flunitrazepam	15.17	0.8252
Methylclonazepam (I.S.)	18.38	1

Table 6
Retention times (RT) and relative retention times (RRT) of other compounds detected by the same HPLC procedure

Substances	RT	RRT
Caffeine	2.09	0.1137
Codeine	2.25	0.1224
Quinine	2.20	0.1197
Tramadol	2.95	0.1605
Desmethylvenlafaxine	3.56	0.1937
Venlafaxine	3.92	0.2133
Propranolol	4.44	0.2416
Benzoylecgonine	5.50	0.2992
Nortryptilline	8.78	0.4777
Amitryptilline	9.82	0.5343
Methadone	10.88	0.5919
Fluoxetine	14.00	0.7617

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