Semiautomated High-Performance Liquid Chromatographic Method for the Determination of Benzodiazepines in Whole Blood

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Abstract

A semiautomated method for the determination of five frequently prescribed benzodiazepines (BZD) (clonazepam, diazepam, flunitrazepam, midazolam, and oxazepam) in whole blood samples by reversed-phase high-performance liquid chromatography following simple online enrichment and clean-up on a short precolumn is described. After precipitation of protein and red cells with a mixture of organic solvents (methanol/acetonitrile, 50:50), the aliquot is centrifuged and the organic upper phase evaporated under a gentle stream of nitrogen. The residue is reconstituted by adding 500 µL of a mixture of phosphate buffer (20mM, pH 2.2) and acetonitrile (70:30, v/v). The sample is then directly introduced into the column-switching column. The precolumn is first washed with phosphate buffer at pH 7.2. Compounds retained on the precolumn are then eluted in the back-flush mode and separated on a C_8 semi-microcolumn (Lichrospher select B, 125 × 3 mm). The BZD studied are determined by a diode-array detector at 254 nm. The method shows excellent linearity between 25 and 1000 ng/mL for clonazepam, flunitrazepam, and midazolam and between 25 and 5000 ng/mL for diazepam and oxazepam. The recoveries are around 80% for clonazepam and oxazepam and around 90% for the three others. Coefficients of variation for between-day and within-day assays are < 15% for low concentrations close to the limit of quantitation and < 5% for high concentrations.

Introduction

Benzodiazepines (BZD) are often used as antianxiety agents in the treatment of psychiatric disorders (1–4). Various researchers have reported total plasma BZD concentration related to both clinical effect and toxicity. A variety of methods for the detection and determination of BZD in biological matrices are described in the literature.

Gas chromatography–mass spectrometry (GC–MS) methods have been frequently reported (5–9). Further studies have been carried out (10–12), and comparisons have been made between GC–MS techniques and various immunoassays (13,14).

High-performance liquid chromatography methods (HPLC) are often applicable to only one drug and its metabolites (15–17). Furthermore, papers reporting screening procedures (18) do not specifically address the problem of clinical and toxicological screening, as very often two or more unrelated drugs are co-administered with benzodiazepines.

The chromatographic theory and method development behind column-switching or online preconcentration techniques have been reviewed and described (19–21).

This paper describes a column-switching technique in which whole blood samples are injected, after protein and red cell precipitation with a mixture of organic solvent, into a BioTrap 500 MS column (extraction column) and are then washed with a mixture of 30mM phosphate buffer (pH 7.2) and acetonitrile mobile phase (94:6, v/v). The drugs retained are then eluted onto a C8 reversed-phase analytical column for determination.

The method presented is rapid and easily automated and allows to determine BZD regularly encountered in clinical and toxicological cases. The column-switching technique offers several advantages: direct injection of biological fluids, nonmanual or robotic clean-up, semiautomated and minimized contact with the infectious biological fluids, on-column enrichment of analytes, and low cost per sample.

Experimental

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 and phosphoric acid were purchased from Merck (Darmstadt, Germany), and

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HPLC-grade acetonitrile was obtained from Romil (Cambridge, England).

Stationary 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 (between pH 2 and pH 11) with a biocompatible external surface (α1-acid glycoprotein) and a hydrophobic internal surface (hydrophobic polymer).

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

The separation was performed using a C_8 reversed-phase semi-microcolumn (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, Oensingen, Switzerland). This method was recently validated (22).

The column switching mobile phase consisted of 30mM phosphate buffer (pH 7.2) and acetonitrile (94:6, v/v). The analytical mobile phase comprised a mixture of 20mM phosphate buffer (pH 2.1) and acetonitrile. The optimized conditions of this technique are detailed in Table I.

Table I. Optimized Conditions

Component	Description
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, 5µm Thermostated at 35°C
Loading	K_2HPO_4, (30mM, pH 7.2) 5 min at 0.6 mL/min
Transfer and analytical separation	A = Acetonitrile (ACN), B = KH_2PO_4, (20mM, ph 2.1) Linear gradient 0 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

Column-switching system

A representative column-switching technique set-up is given in Figure 1. The online system consisted of two quaternary pumps (model HP 1100) and two columns connected by an HP 1100 high-pressure six-port valve in back-flush configuration. The chromatographic system was equipped with a diode-array detector, an automatic injector, and an autosampler.

In extraction position, pump A pumped the extraction mobile phase through the autosampler, into which the sample was injected. A filterholder was inserted after the autosampler with a 2-µm biocompatibility filter. After passing through the filter, the sample was transported to the extraction column via the six-port valve. During this time, analytical pump B pumped the analytical mobile phase through the column via the six-port valve.

In elution position, the mobile phase from pump A (the extraction mobile phase) went to waste. The mobile phase from pump B (analytical mobile phase) back-flushed from the extraction column to the analytical column.

The strong elution power of the second mobile phase delivered by pump B caused analyte desorption from the extraction column. The change in flow direction (back-flush) caused additional analyte concentration.

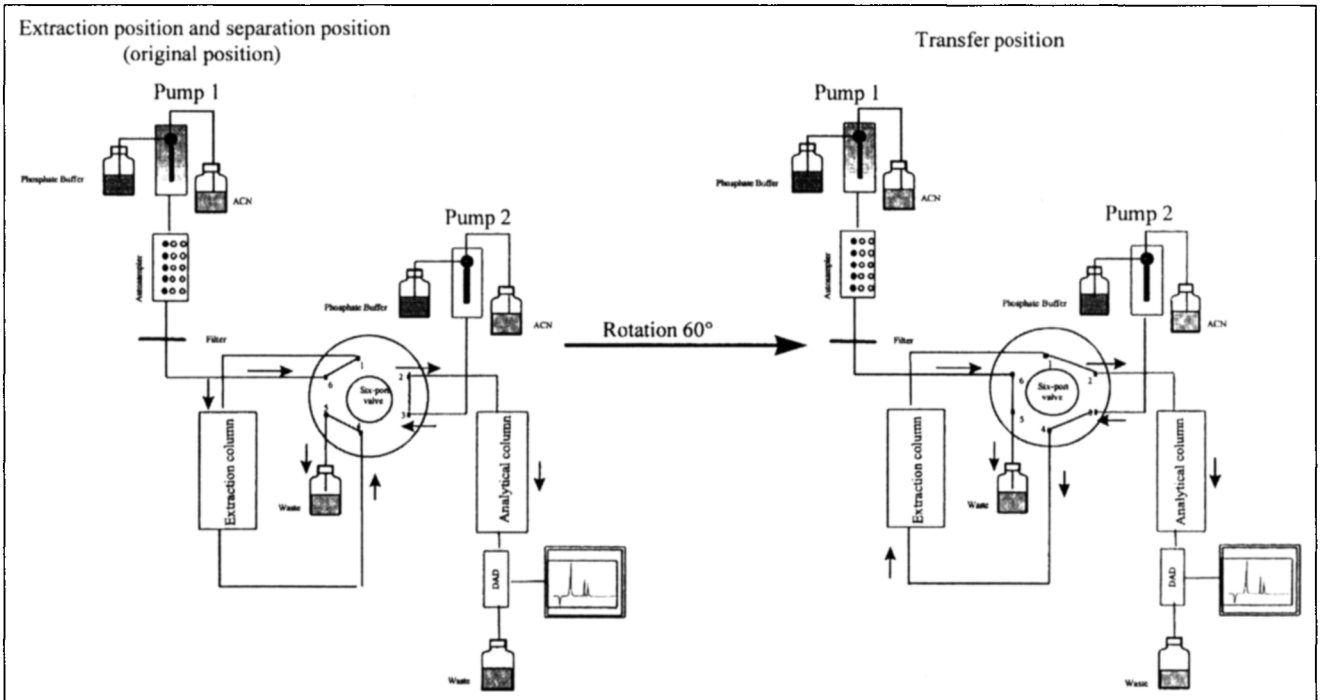

Figure 1. Schematic representation of the column-switching set-up. Adapted from reference 23.

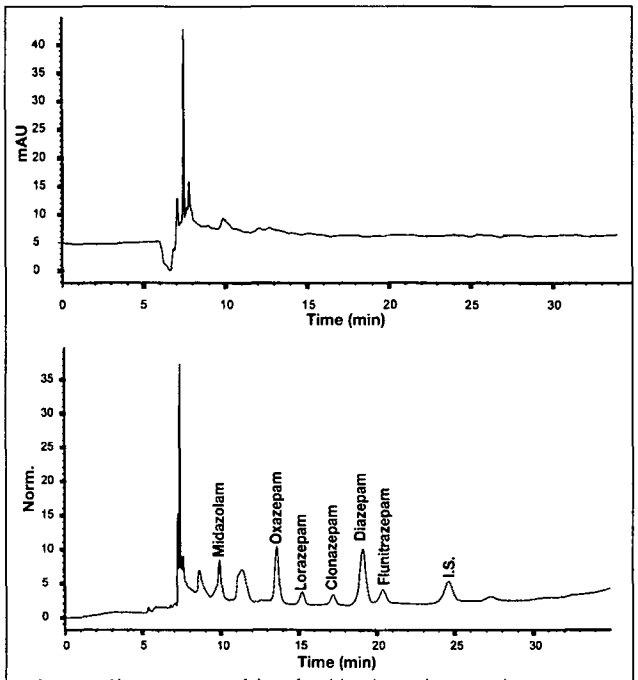

Figure 2. Chromatogram of drug-free blood sample (top). Chromatogram of spiked blood with clonazepam (300 ng/mL), flunitrazepam (300 ng/mL), midazolam (300 ng/mL), diazepam (1000 ng/mL), and oxazepam (1000 ng/mL) (bottom), and methylclonazepam (2000 ng/ml I.S.). Extraction conditions: injection, 50 mL of blood sample (after precipitation) on BioTrap 500 MS extraction (20 x 4-mm i.d); mobile phase, acetonitrile/phosphate buffer (pH 7.2, 0.3M). Analytical conditions: C8 reversed-phase column, Lichrospher Select B (125 x 3-mm i.d); mobile phase, acetonitrile/phosphate buffer (pH 2.1, 0.2M) (35:65, v/v) at a flow rate of 0.3 mL/min UV detection at 254 nm.

Table II. Calibration Data for the Five Benzodiazepines (n = 3)

	Range (µg/mL)	Coefficient of correlation r	Line $Y = ax + b$
Clonazepam	0.025–1.0	0.995	$0.0013x - 0.074$
Diazepam	0.025–5.0	0.999	$0.002x + 0.048$
Flunitrazepam	0.025–1.0	0.996	$0.0028x - 0.14$
Midazolam	0.025–1.0	0.997	$0.0017x + 0.18$
Oxazepam	0.025–5.0	0.995	$0.0015x - 0.34$

In separation position, the valve was switched back to its original position (extraction position), the analyte fraction caused transfer into the analytical column, and analyte separation then took place in a conventional manner.

Eluent absorbance was monitored at 254 nm. HP ChemStation (Hewlett-Packard Software no. G2170AA) was used for instrument control, data acquisition, and data handling.

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. Stock solutions were stored at –20°C and remained stable for at least 24 months.

Biological standards were prepared at the required concentrations by diluting the appropriate aliquots of the stock solutions with clean drug-free blood between 25 and 5000 ng/mL for both oxazepam and diazepam and between 25 and 1000 ng/mL for clonazepam, flunitrazepam, and midazolam (Figure 2).

Phosphate buffer

The extraction phosphate buffer (pH 7.5, 30mM) was prepared by transferring 2.7 mL of 1M KH_2PO_4 and 9.9 mL of 1M K_2HPO_4 into a 1000-mL volumetric flask and making up to volume with distilled water. The analytical phosphate buffer (pH 2.1, 20mM) was prepared by transferring 12.7 mL of 1M KH_2PO_4 and 22.3 mL of 1M H_3PO_4 into a 1000-mL volumetric flask and making up to volume with distilled water. Buffer solutions were always freshly prepared and filtered through a 0.45-µm filter (Supelco, Bellefonte, PA) immediately before use.

Sample preparation

Whole blood samples (1 mL) were spiked with the different mentioned benzodiazepines at the desired concentrations and with 20 µL of the appropriate internal standard (methylclonazepam, 100 µg/mL). Spiked samples were mixed with 2 mL of a mixture of organic solvent (methanol/acetonitrile, 1:1), vortex mixed, and centrifuged for 10 min at 5000 rpm. The supernatant was evaporated to dryness and dissolved in 500 µL of analytical mobile phase, then placed into glass vials. Fifty microliters was processed online as described.

Table III. Repeatability and Reproducibility (n = 6)

	Clonazepam		Flunitrazepam		Midazolam		Oxazepam		Diazepam	
Concentration (ng/mL)	repeat.* CV%	repro. CV%	repeat. CV%	repro. CV%	repeat. CV%	repro. CV%	repeat. CV%	repro. CV%	repeat. CV%	repro. CV%
50	15.2	14.6	12.8	13.5	13.9	9.9	–†	–	–	–
250	–	–	–	–	–	–	4.8	4.7	4.4	4.1
300	5.2	6.7	2.4	3.5	4.9	3.4	–	–	–	–
500	4.2	3.6	2.0	2.3	4.5	3.0	–	–	–	–
3000	–	–	–	–	–	–	3.8	3.1	4.0	3.7
5000	–	–	–	–	–	–	2.1	3.0	2.9	2.0

* Abbreviations: repeat., repeatability and repro., reproducibility.
† Not analyzed at this concentration.

Results

Chromatography

The five benzodiazepines were well separated, and the whole procedure allowed an extremely clean chromatographic trace (Figure 2).

Linearity

Detector response linearity was evaluated by preparing five triplicate calibrations covering the following concentration ranges: 25 to 1000 ng/mL for clonazepam, flunitrazepam, and midazolam and 25 to 5000 ng/mL for diazepam and oxazepam. Linear regression lines were obtained by plotting peak-area ratios (the compound peak area divided by one of the internal standards, see Table II).

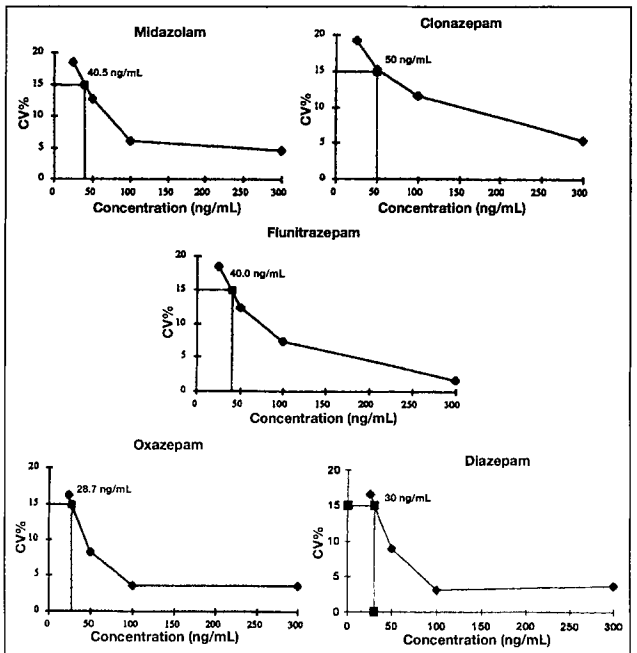

Figure 3. Curve determination of LOQ values.

Precision

Within-day reproducibility or repeatability (see Table III) was evaluated by replicate analysis (n = 6) of pooled blood at three different concentrations: 250, 3000, and 5000 ng/mL for diazepam and oxazepam and 50, 300, and 500 ng/mL for the three others on the same day. The coefficients of variation (CV) obtained are shown in Table III.

Between-day reproducibility or reproducibility (Table III) was determined by replicate analysis (n = 6), over three days, at the same concentrations as for repeatability.

Assay detection limits

Limit of detection (LOD). The LOD, defined as the lowest analyte concentration 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 spiked blood with BZD in decreasing concentrations. LOD was determined to be around 15 ng/mL for clonazepam, flunitrazepam, and midazolam and 10 ng/mL for diazepam and oxazepam.

Limit of quantitation (LOQ). The LOQ (n = 6) is the lowest concentration that can be measured on the standard curves with acceptable reproducibility (CV < 15%) (Figure 3). The lower pratical LOQ was 50 ng/mL for clonazepam, below 40 ng/mL for flunitrazepam and midazolam, and 30 ng/mL for diazepam and oxazepam.

Recovery

Recoveries of the five benzodiazepines studied were determined by comparing the peak areas of the spiked blood samples and of the reference samples at six different concentrations. The reference samples were injected directly into the analytical column and the spiked blood samples were injected, after precipitation with a mixture of organic solvent, into the extraction column coupled to the analytical column through the switching valve.

As showed in Table IV, the method gives acceptable values for recoveries. The observed recovery is higher than 90% for all BZD, except clonazepam and oxazepam (around 80%).

Table IV. Recoveries Obtained with Spiked Plasma Samples (n = 6)

Amount added (ng/mL)	Clonazepam mean recovery (%)	Flunitrazepam mean recovery (%)	Midazolam mean recovery (%)	Oxazepam mean recovery (%)	Diazepam mean recovery (%)
25	70.0	83.1	95.0	67.1	84.2
50	67.0	88.3	101.7	-*	-
100	76.7	87.0	113.0	-	-
250	-	-	-	72.3	88.3
300	76.7	84.0	100.3	-	-
500	82.8	105.7	101.3	76.3	88.3
800	81.0	97.7	107.7	-	-
1000	80.0	96.0	113.3	74.3	91.0
2000	-	-	-	74.3	104.0
3000	-	-	-	79.3	103.0
5000	-	-	-	89.3	102.0

* Not analyzed at this concentration.

Application to Postmortem Blood Samples

Generally, postmortem 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 four benzodiazepines (desalkylflurazepam, flurazepam, desmethyldiazepam, and oxazepam) and one antidepressant (venlafaxine) (Figure 4). The measured concentrations were as follows: desalkylflurazepam, 42 ng/mL; flurazepam, 52 ng/mL; desmethyldiazepam, 310 ng/mL; and oxazepam, 1150 ng/mL.

In the second blood sample, also obtained from a deceased person, the following substances were clearly identified: benzodiazepines (demoxepam and desmethyldiazepam), one antidepressant (amitriyptiline), propranolol, and tramadol (Figure 5). The following concentrations were determined: demoxepam, 995 ng/mL; desmethyldiazepam, 1095 ng/mL; and amitryptiline, 605 ng/mL.

In the third blood sample obtained from a deceased subject, two benzodiazepines (lorazepam and oxazepam) were detected (Figure 6), and the following concentrations were measured: lorazepam, 905 ng/mL and oxazepam, 2075 ng/mL.

Conclusions

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

Satisfactory validation data were achieved for linearity, precision, and recovery. The LOQ allows measurement of therapeutic concentrations for most benzodiazepines and toxic concentrations for flunitrazepam. For the therapeutic concentration of flunitrazepam, the supernatant is evaporated to dry-

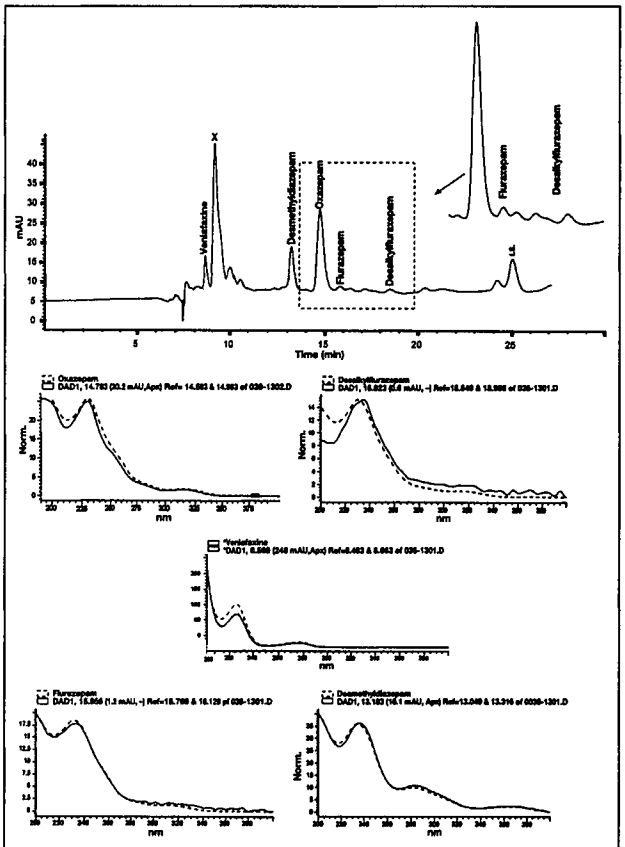

Figure 4. Chromatograms and UV spectra of blood sample obtained from a deceased subject. Extraction conditions: injection, 50 µL of blood sample (after precipitation), on BioTrap 500 MS extraction (20 x 4-mm i.d); mobile phase, acetonitrile-phosphate buffer (pH 7.2, 0.3M). Analytical conditions: C8 reversed-phase column, Lichrospher Select B (125 x 3-mm i.d); mobile phase, acetonitrile/phosphate buffer (pH 2.1, 0.2M) UV detection at 254 nm

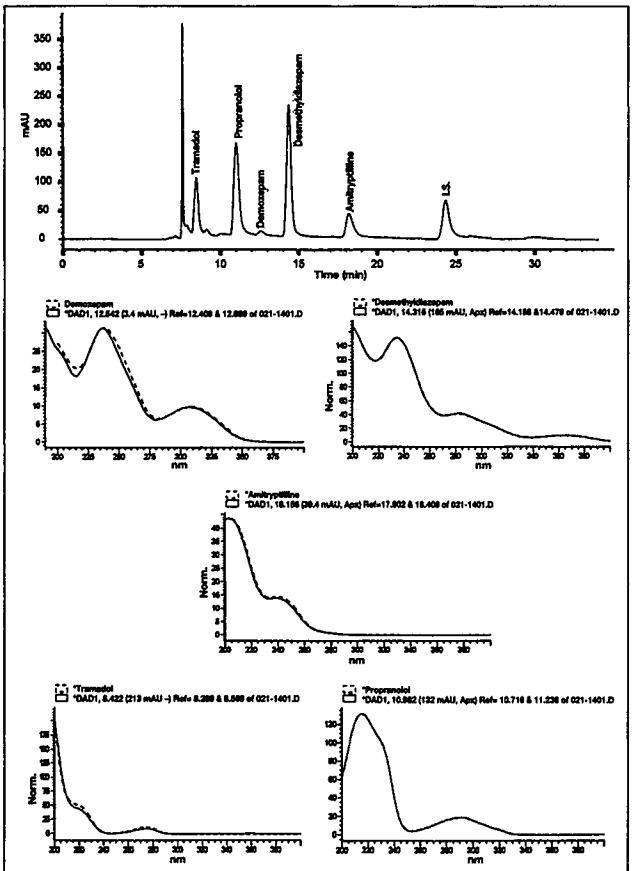

Figure 5. Chromatograms and UV spectra of blood sample obtained from a deceased subject. Extraction conditions: injection, 50 µL of blood sample (after precipitation), on BioTrap 500 MS extraction (20 x 4-mm i.d); mobile phase, acetonitrile/phosphate buffer (pH 7.2, 0.3M). Analytical conditions: C8 reversed-phase column, Lichrospher Select B (125 x 3-mm i.d); mobile phase, acetonitrile/phosphate buffer (pH 2.1, 0.2M); UV detection at 254 nm

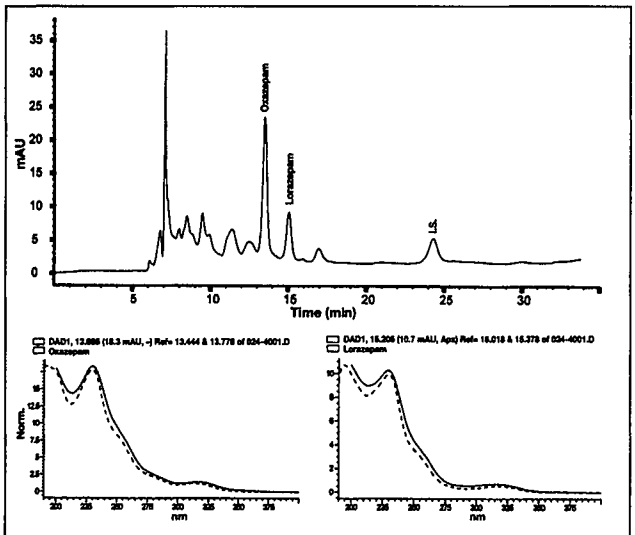

Figure 6. Chromatograms and UV spectra of blood sample obtained from a deceased subject. Extraction conditions: injection, 50 µL of blood sample (after precipitation), on BioTrap 500 MS extraction (20 x 4-mm i.d); mobile phase, acetonitrile/phosphate buffer (pH 7.2, 0.3M). Analytical conditions: C8 reversed-phase column, Lichrospher Select B (125 x 3-mm i.d); mobile phase, acetonitrile/phosphate buffer (pH 2.1, 0.2M); UV detection at 254 nm

ness and dissolved in 100–150 µL of mobile phase.

The use of a column-switching technique allows to minimization of contact with infectious biological fluids like blood and plasma.

Finally, the proposed method is not only suitable for determining benzodiazepine in whole blood samples, but can also be applied to other drugs such as antidepressants.

References

1. R.I. Shader and D.J. Greenblatt. Clinical implication of benzodiazepines in pharmacokinetics. *Am. J. Psych.* **134:** 652–656 (1977).
2. K. Jinno, M. Taniguchi, and M. Hayashido. Solid phase micro extraction coupled with semi-microcolumn high-performance liquid chromatography for the analysis of benzodiazepines in human urine. *J. Pharm. Biomed. Anal.* **17:** 1081–1091 (1998).
3. E. Tanaka, M. Terada, S. Misawa, and C. Wakasugi. Simultaneous determination of twelve benzodiazepines in human serum using a new reversed-phase chromatographic column on a 2-microns porous microspherical silica gel. *J. Chromatogr. B* **682:** 173–178 (1996).
4. J.B. Roberts and J.A. Tafuri. *Clinical Management of Poisoning and Drug Overdose*, L.M. Haddad and J.F. Winchester, Eds. W.B. Saunder, Philadelphia, PA, 1990, pp 800–820.
5. K. Kudo, T. Nagata, K. Kimura, T. Imamura, and M. Noda. Sensitive determination of diazepam and N-desmethyldiazepam in human material using capillary gas chromatography–mass spectrometry. *J. Chromatogr.* **431:** 351–359 (1988).
6. N. De Giovanni and M. Chiarotti. Analysis of benzodiazepines. II. High-performance liquid chromatography–fluorescence detection after molecular rearrangement to acridanones. *J. Chromatogr.* **428:** 321–329 (1988).
7. H. Maurer and K. Pfleger. Identification and differentiation of benzodiazepines and their metabolite in urine by computerized gas-chromatography–mass spectrometry. *J. Chromatogr.* **422:** 85–101 (1987).
8. M. Japp, K. Garthwaite, A.V. Geeson, and M.D. Osselton. Collection of analytical data for benzodiazepines and benzophenones. *J. Chromatogr.* **439:** 317–339 (1988).
9. A.J.H. Louter, E. Bosma, J.C.A. Schipperon, J.J. Vreuls, and U.A.Th. Brinkman. Automated on-line solid-phase extraction gas-chromatography with nitrogen phosphorus detection: determination of benzodiazepines in human plasma. *J. Chromatogr. B* **689:** 35–43 (1997).
10. D.A. Black, G.D. Clark, V.M. Haver, J.A. Garbin, and A.J. Saxon. Analysis of urinary benzodiazepines using solid-phase extraction and gas chromatography–mass spectrometry. *J. Anal. Toxicol.* **18:** 185 (1994).
11. C. Moore, G. Long, and M. Marr. Confirmation of benzodiazepines in urine as trimethylsilyl-derivatives using gas-chromatography–mass spectrometry. *J. Chromatogr. B* **655:** 132–137 (1994).
12. K.M. Hold, D.J. Crouch, D.E. Rollins, D.G. Wilkins, D.V. Canfield, and R.A. Maes. Determination of alprazolam and α-hydroxyalprazolam in human plasma by gas chromatography/negative-ion chemical ionization mass spectrometry. *J. Mass Spectrom.* **31:** 1033–1038 (1996).
13. R.L. Fitzgerald, P.A. Rexin, and D.A. Herold. Detection benzodiazepines: immunoassays compared with negative chemical ionization gas chromatography/mass spectrometry. *Clin. Chem.* **40:** 373–380 (1994).
14. T. Nishikawa, H. Ohtani, D.A. Herold, and R.L. Fitzgerald. Comparison of assay methods for benzodiazepines in urine, a receptor assay, two immunoassays and gas chromatography–mass spectrometry. *Am. J. Clin. Pathol.* **107:** 345–352 (1997).
15. T.B. Vree, A.M. Boars, Y.A. Hekster, and E. Van der kleijn. Simultaneous determination of chlordiazepoxid and its metabolites in human plasma and urine by means of reversed-phase high-performance liquid chromatography. *J. Chromatogr.* **224:** 519–525 (1981).
16. L. Wen-Nuei. Determination of clonazepam in serum by high pressure liquid chromatography. *Ther. Drug Monit.* **9:** 337–342 (1987).
17. M. Sajgo. Determination of tofisopam in serum by high-performance liquid chromatography. *J. Chromatogr.* **317:** 303–307 (1981).
18. P. Mura, A. Piriou, P. Fraillon, Y. Papet, and D. Reiss. Screening procedure for benzodiazepines in biological fluids by high-performance liquid chromatography using a rapid scanning multichannel detector. *J. Chromatogr.* **416:** 303–310 (1987).
19. D. Westerlund. Direct injection of plasma into column liquid chromatographic system (Review). *Chromatographia* **24:** 155–164 (1987).
20. J.B. Lecaillon, N. Febvre, and C. Souppart. Influence of solute polarity in column-switching chromatography for the assay of drug in plasma and urine (Review). *J. Chromatogr.* **317:** 493–506 (1984).
21. A. El Mahjoub and C. Staub. High-performance liquid chromatographic method for the determination of benzodiazepines in plasma or serum using the column-switching technique. *J. Chromatogr.* **742:** 381–390 (2000).
22. A. El Mahjoub and C. Staub. Simultaneous determination of benzodiazepines in whole blood or serum by HPLC/DAD with a semi-micro column. *J. Pharm. Biomed. Anal.* **23:** 447–458 (2000).
23. J. Henderson and A. Grahn. Determination of drugs by direct injection of plasma into biocompatible extraction based on protein-entrapped hydropholic phases. *J. Chromatogr.* **660:** 119–129 (1994).

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