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A rapid solid-phase extraction and HPLC/DAD procedure for the simultaneous determination and quantification of different benzodiazepines in serum, blood and post-mortem blood

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Summary. A rapid and quantitative method for the determination of benzodiazepines using high-performance liquid chromatography (HPLC) with diode-array detection (DAD) is reported. The drugs were extracted from serum, blood or post-mortem blood using C_{18} extraction columns. Brotizolam was used as internal standard. Experiments with spiked serum/blood samples resulted in recoveries between 75% and 94% for all investigated benzodiazepines. Excellent linearity was obtained over the concentration range 5–1500 ng benzodiazepine/ml. The limit of detection was approximately 2 ng/ml. The detection of low therapeutic serum levels of highly potent benzodiazepines is also possible.

Key words: Benzodiazepines – Serum, blood, post-mortem blood – Solid-phase extraction – HPLC/DAD

Zusammenfassung. Eine schnelle Methode zur quantitativen Bestimmung von Benzodiazepinen mittels Hochleistungsflüssigkeitschromatographie (HPLC) mit Dioden-Array-Detektion (DAD) wird beschrieben. Die Substanzen werden aus Serum, Vollblut oder Leichenblut mit Hilfe von C_{18}-Extraktionssäulen extrahiert. Brotizolam wird als interner Standard eingesetzt. Versuche mit aufgestockten Serum-/Blutproben führten für sämtliche untersuchten Benzodiazepine zu Wiederfindungsraten zwischen 75% und 94%. Eine ausgezeichnete Linearität wurde über den Konzentrationsbereich von 5–1500 ng Benzodiazepin/ml beobachtet. Die Nachweisgrenze lag für alle Substanzen bei annähernd 2 ng/ml. Auch für die niedrig dosierten Benzodiazepine ist die Erfassung der therapeutischen Konzentrationsbereiche gewährleistet.

Schlüsselwörter: Benzodiazepine – Serum, Blut, Leichenblut – Festphasen-Extraktion – HPLC/DAD

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Introduction

Benzodiazepines are used as anticonvulsants, anxiolytics, hypnotics and muscle relaxants with different durations of action [1–3]. Screening and detection can be necessary under therapeutic aspects as well as in connection with forensic toxicology and traffic medicine [4]. Current methods for the determination of benzodiazepines available in clinical or forensic chemistry include thin-layer chromatography (TLC) [5, 6], gas chromatography often coupled with mass spectrometry (GC/MS) [7–11], immunological methods [12–16] or high-performance liquid chromatography (HPLC) [11, 17–22]. The latter technique is particularly suitable for the quantitative determination of substances with intense UV absorption, such as the 1,4-benzodiazepines, particularly since some of these compounds exhibit thermal instability [23, 24]. Although its value as a screening method is still controversial, the combination with diode-array detection has to be considered as a highly effective and selective screening method [25, 26]. Solid-phase extraction performed on a bonded silica phase is an alternative to liquid-liquid extraction for the isolation of drugs. Ease of operation including elimination of time consuming steps

Table 1. Therapeutic and toxic serum concentrations [3, 28]

Benzodiazepine	Therapeutic range (mg/l)	Toxic range (mg/l)
Midazolam	0.08 –0.25	–
Bromazepam	0.08 –0.17	0.25–0.50
Oxazepam	1.00 –2.00	3.00–5.00
Nordazepam	0.20 –0.80	2.00
Tetrazepam	0.30 –1.00	–
Flunitrazepam	0.005–0.015	0.05
Triazolam	0.002–0.02	–
Diazepam	0.50 –0.75	1.50–3.00

and excellent recoveries and reproducibility have been reported [19, 20, 27].

The following report describes a solid-phase extraction procedure for the following 8 benzodiazepines: bromazepam, diazepam, flunitrazepam, midazolam, nordazepam, oxazepam, tetrazepam and triazolam, which have a wide range of therapeutic concentrations (Table 1). For quantitative determination with HPLC/DAD brotizolam was used as internal standard. With this procedure other benzodiazepines and their metabolites can also be determined.

Materials and methods

Chemicals. Methanol (Uvasol, Merck Darmstadt), acetonitrile (HPLC grade, Merck Darmstadt), acetone (p.a., Merck Darmstadt), water (HPLC grade, Baker Groß-Gerau), inorganic chemicals: boric acid, sodium tetraborate, potassium dihydrogen phosphate, orthophosphoric acid, sodium hydroxide, hydrochloric acid (p.a., Merck Darmstadt). Worldwide Monitoring Clean Up C_{18} end-capped extraction columns (100 mg) were purchased from Amchro (Sulzbach/Taunus). Benzodiazepines were obtained commercially and 0.1% stock solutions were prepared in methanol. Internal standard: 25 µg brotizolam/ml methanol.

Borate buffer (pH9): 835 ml solution A (12.37 g boric acid + 100 ml sodium hydroxide (1 mol/L) with sodium tetraborate (0.05 mol/l) ad 1 l) + 165 ml solution B (hydrochloric acid (0.1 mol/l)).

Instrumentation and chromatographic parameters. The extraction columns were positioned on a Vacuum-Manifold (Amchro).

Chromatographic analysis was performed under isocratic conditions at a flow rate of 1.3 ml/min on a Perkin Elmer system consisting of a Series 1 LC Pump, a Kontrosorb 10 RP 18 column (250 × 4.6 mm i.d.), a Perkin Elmer LC-480 Auto Scan Diode Array Detector with a 16 mm cell and a PC station with software (LC-DES plus) from Perkin Elmer.

The mobile phase was a mixture of 156 g acetonitrile and 340 g aqueous buffer (4.8 g of 85% orthophosphoric acid and 6.66 g of potassium dihydrogen phosphate in 1 l water, adjusted to pH 2.3).

Extraction procedure. Serum, blood (2 ml) or post-mortem blood (2 g) were spiked with different benzodiazepines and 20 µl of internal standard. Spiked blood samples were mixed with 2 ml acetone, vortexed and centrifuged for 5 min at 2000 rpm. The supernatant was evaporated to dryness and dissolved in 2 ml borate buffer. Spiked serum samples were prepared for solid-phase extraction by adding 2 ml borate buffer.

The extraction columns were conditioned by washing with 2 ml methanol followed by 2 ml water and 1 ml borate buffer. Prepared samples (2 ml or 4 ml) were applied to the columns and drawn through by a vacuum with a flow rate of approximately 1 ml/min. The columns were washed with 1 mL water followed by 1 ml 15% methanol in water and dried by centrifugation of the columns (5 min, 2000 rpm). The benzodiazepines were eluted with 1 ml methanol and collected in a vial. The eluate was evaporated to dryness at 50°C under a stream of nitrogen. The residue was dissolved in 20 µl methanol and a 10 µl aliquot was subjected to HPLC analysis.

Results and discussion

For our experiments we have spiked serum, blood and post-mortem blood with 2 different benzodiazepine solutions. Solution A contained bromazepam, oxazepam, nordazepam and flunitrazepam, solution B contained midazolam, tetrazepam, triazolam and diazepam. Figure

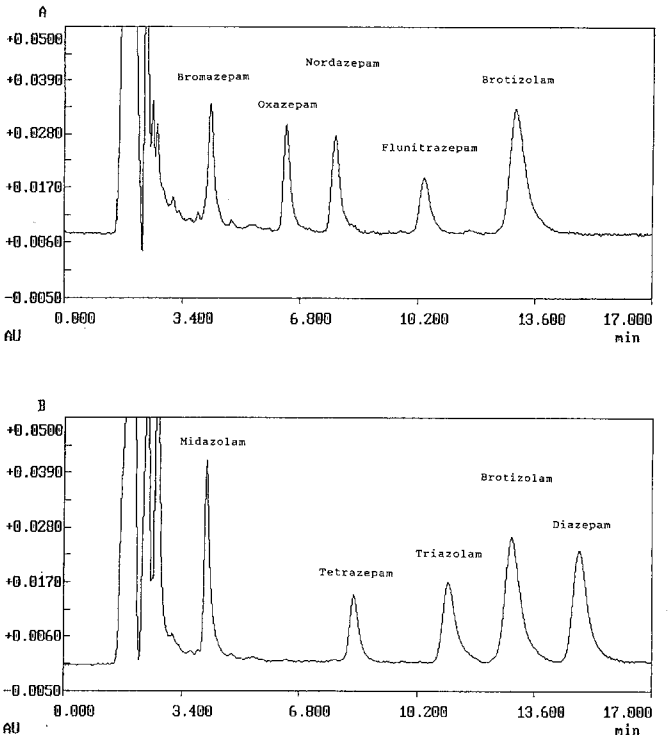

Fig. 1A, B. Chromatograms of spiked blood samples with 100 ng benzodiazepine/ml and 250 ng internal standard brotizolam at 220 nm

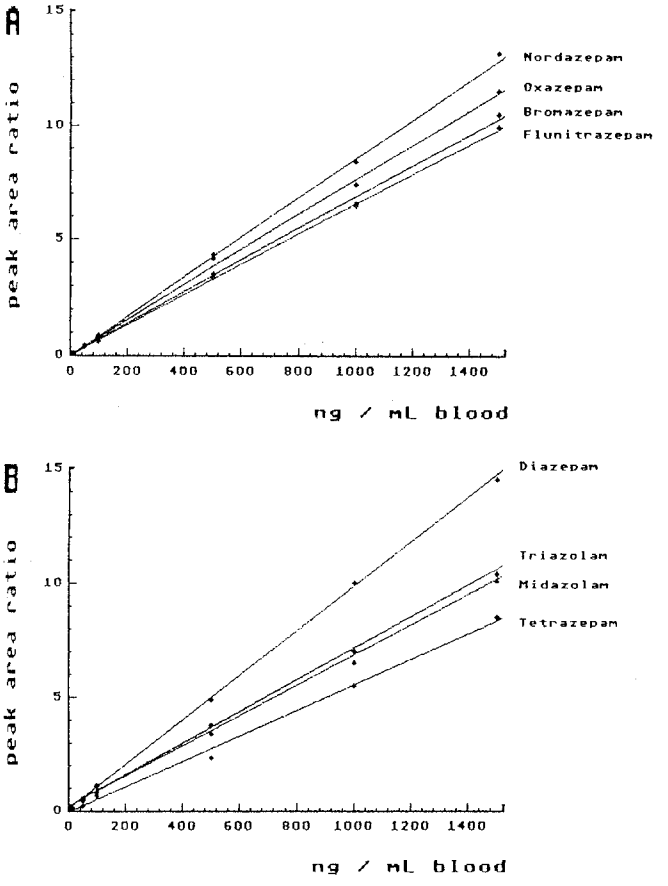

Fig. 2A, B. Calibration curves with 5 replicates/substance and concentration

Table 2. Precision data with spiked blood extracts with 5 replicates/substance

	Bromazepam		Oxazepam		Nordazepam		Flunitrazepam		Midazolam		Tetrazepam		Triazolam		Diazepam	
Added (ng/ml)	10	500	10	500	10	500	10	500	10	500	10	500	10	500	10	500
Found (ng/ml) ± SD	9.7 ± 0.78	488 ± 35.4	10.7 ± 1.34	483 ± 28.8	10.5 ± 0.74	480 ± 31.7	9.7 ± 1.15	477 ± 40.2	10.6 ± 0.98	489 ± 47.8	10.8 ± 1.04	516 ± 50.2	9.0 ± 1.15	471 ± 48.1	11.1 ± 1.13	523 ± 24.4
CV %	7.8	7.1	13.4	5.8	7.4	6.3	11.5	8.0	9.8	9.6	10.4	10.0	11.5	9.6	11.3	4.9
Recovery (%) ± SD	80.1 ± 2.4	83.1 ± 2.3	79.8 ± 3.5	81.9 ± 3.2	86.1 ± 3.3	90.6 ± 2.7	80.8 ± 3.1	84.1 ± 2.9	80.4 ± 3.1	84.4 ± 2.7	76.7 ± 3.1	81.4 ± 2.8	74.9 ± 1.9	79.8 ± 2.4	90.0 ± 3.0	94.1 ± 2.2
r: coefficient of correlation (calibration curve)	0.996		0.994		0.993		0.996		0.996		0.997		0.996		0.994	

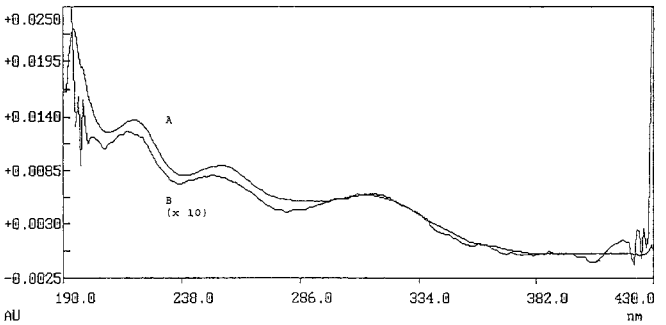

Fig. 3. UV-spectrum as additional identification mark from *A*: 100 ng flunitrazepam and *B*: 10 ng flunitrazepam (× 10) extracted from blood

1 shows the chromatograms of spiked blood sample extracts with 100 ng benzodiazepine/ml detected at 220 nm. For quantification the peak area ratios (benzodiazepine: internal standard) were calculated. Excellent linearity was obtained over the range 5–1500 ng/ml blood for all drugs investigated (Fig. 2). The coefficients of correlation for the calibration curves ranged from 0.993 to 0.997. Recoveries and reproducibility of the method were measured by analysing 5 replicates of 2 blood samples assayed to contain 10 ng and 500 ng benzodiazepines/ml (Table 2). The absolute recoveries (means ± SD) determined with external standards ranged from 79.8% ± 3.5% to 94.1% ± 2.2% for the high concentration samples and from 74.9% ± 1.9% to 90.0% ± 3.0% for the low concentration samples. The day-to-day precision CV's ranged from 4.9% to 10.0% at the higher levels and from 7.8% to 13.3% at the lower levels. Using the routine procedure described, the minimal detectable concentrations of the benzodiazepines were 1–2 ng/ml blood or serum with a signal-noise ratio 3:1. The lowest amount that yielded a spectrum with diode-array detection was approximately 10 ng (Fig. 3). For lower amounts a definite confirmation by GC/MS was possible.

This procedure for the simultaneous identification and quantification of various benzodiazepines is based on a combination of 2 processes. The clean up C_{18} column is used to achieve high recovery of the drugs from biological fluids and the HPLC/DAD system is used to identify the compounds.

Optimum absorption of the benzodiazepines on the C_{18} matrix was obtained by adjusting the sample to pH 9. To produce highly purified extracts the columns were first washed with distilled water, then with 15% aqueous methanol to remove interfering polar components. These washing steps did not reduce the recoveries of benzodiazepines but improved the purity of the extracts better than washing with water alone. These extracts can also be used for GC/MS analysis.

The identification and quantification with the HPLC/DAD system showed a high accuracy for all investigated benzodiazepines over the concentration range 5–1500 ng/ml. HPLC in combination with diode-array detection must be considered as a highly effective screening method. The 1.4-benzodiazepines are compounds with intense UV absorption and the UV spectra are therefore good

Table 3. Retention times and UV-maxima (in methanol)

A	RT (min)	UVmax (nm)			B	RT (min)	UVmax (nm)		
Bromazepam	4.23	234	314		Midazolam	3.99	219		
Oxazepam	6.42	229	315		Tetrazepam	8.34	240	284	346
Nordazepam	7.80	232	279		Triazolam	11.07	221	280	
Flunitrazepam	10.41	219	253	313	Brotizolam	13.02	240		
Brotizolam	13.02	240			Diazepam	14.94	232		

Table 4. Other benzodiazepines detected by the same HPLC procedure

Benzodiazepine	Retention-time (min)	Benzodiazepine	Retention-time (min)
Alprazolam	10.29	Hydroxyethylflurazepam	7.29
7-Aminoclonazepam	2.50	Hydroxymidazolam	3.57
7-Aminoflunitrazepam	3.00	Ketazolam	14.50
7-Aminonitrazepam	2.08	Lorazepam	6.90
Camazepam	21.66	Lormetazepam	11.46
Chlordiazepoxide	2.85	Medazepam	4.49
Clobazam	11.31	Metaclazepam	7.13
Clonazepam	7.96	Nitrazepam	6.43
Clotiazepam	11.99	Noraminoflunitrazepam	2.34
Demoxepam	4.72	Norchlordiazepoxide	4.83
Desalkylflurazepam	9.24	Norclobazam	7.22
Estazolam	7.85	Norflunitrazepam	6.64
Flurazepam	4.04	Oxazolam	3.53
3-Hydroxybromazepam	2.99	Temazepam	10.01

identification characteristics in addition to the retention times for a screening procedure. An additional advantage of the diode-array detection is the post-run selection of wavelengths that provides the optimum signal-noise ratio for any particular compound (UV maxima in Table 3). Other benzodiazepines and their metabolites can be detected by the same procedure (Table 4).

In summary, an efficient extraction of benzodiazepines and their metabolites from serum, blood or post-mortem blood with a HPLC/DAD analysis is reported. The procedure, established as a routine method in our laboratory, is relatively simple, reproducible and sensitive and produces results within 45 min. The detection of low therapeutic serum levels of highly potent benzodiazepines is also ensured.

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