Journal of Pharmaceutical & Biomedical Analysis
Vol. 10, No. 5, pp. 317–322, 1992
Printed in Great Britain

0731–7085/92 $5.00 + 0.00

Simultaneous determination of common benzodiazepines in blood using capillary gas chromatography

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Abstract: Blood samples were extracted with *n*-butyl acetate, and the extracts analysed by capillary gas chromatography using DB-1 and DB-1701 capillary columns with electron-capture detection. The DB-1701 column was found to give better separation of different benzodiazepines (BZDs). Recoveries ranged from 79 to 98%. Detection limits ranged from 0.005 to 0.015 μM for triazolam and flunitrazepam, and from 0.02 to 0.1 μM for other BZDs. Data on accuracy and precision are given for diazepam, desmethyldiazepam, flunitrazepam and nitrazepam.

Keywords: *Benzodiazepines; blood; capillary gas chromatography.*

Introduction

Benzodiazepines (BZDs) are the most frequently used sedative and hypnotic drugs in western countries [1]. BZDs also are among the most commonly misused drugs [2]. Blood samples are frequently analysed for BZDs both in clinical and in forensic toxicology, but lethal intoxications by BZDs are seldom [1–3]. In forensic cases of impairment by drugs, such as driving under influence of drugs, BZDs are among the most frequent causes of impairment [4–7].

Simultaneous detection and quantification of several BZDs can be performed using high-performance liquid chromatography [8–10], gas chromatography [11–14], or gas chromatography–mass spectrometry [15]. BZDs are most commonly extracted by an organic solvent, which is evaporated in order to concentrate the extract, and the residue is dissolved in a small volume of solvent [8–11, 13–15].

The therapeutic levels of flunitrazepam and triazolam are much lower than for other BZDs [16], therefore, the detection limits for flunitrazepam and triazolam should be very low compared with other BZDs.

During the last few years there has been a large increase in the number of samples analysed for BZDs in the authors' laboratory. A simple method for analysis of BZDs therefore has been developed, based upon a simple extraction procedure with no preconcentration of drugs followed by electron-capture GC using medium polar and nonpolar columns. The method is used for screening and confirmation of diazepam, desmethyldiazepam, flunitrazepam, nitrazepam, clonazepam, flurazepam, desalkylflurazepam, oxazepam and triazolam, and for the determination of the most frequently detected BZDs, namely: diazepam; desmethyldiazepam; flunitrazepam and nitrazepam.

Experimental

Chemicals

Diazepam, desmethyldiazepam, nitrazepam, flunitrazepam, flurazepam, desalkylflurazepam and clonazepam were obtained from Hoffmann-La Roche (Basle, Switzerland), triazolam and alprazolam from Upjohn (Puurs, Belgium), methylnitrazepam from Apothekernes Laboratorium (Oslo, Norway), oxazepam from Ferrosan (Copenhagen, Denmark) and prazepam from Orgamol (Evionnaz, Switzerland). Sodium dihydrogenphosphate and disodium hydrogenphosphate were purchased from Merck (Darmstadt, Germany) and *n*-butyl acetate from Rathburn (Walkerburn, Scotland, UK).

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Tubes and pipettes

Extractions were performed in silanized glass tubes with Teflon caps.

Disposable glass pipettes were used for pipetting blood samples and *n*-butyl acetate. Eppendorf Multipipettes model No. 4780 (Eppendorf, Hamburg, Germany) were used for pipetting buffer and internal standard solutions.

Biological materials

Blood for evaluation of the method was obtained from a blood bank. Sodium fluoride (1%) was added, and the blood was stored in glass containers. Blood samples were spiked with aqueous solutions of BZDs, and kept frozen at −20°C. Stock solutions were prepared in methanol.

Calibration standards

Calibration standards were prepared by spiking blood obtained from a blood bank with aqueous solutions of BZDs. The final blood concentrations of drugs are shown in Table 1. Calibration standards were stored at −20°C for up to 3 months. Stock solutions were prepared using methanol as solvent.

Internal standards

When analysing on a DB-1701 column, an aqueous solution of alprazolam (12.5 μM) was used for analysis of triazolam, and an aqueous solution of methylnitrazepam (12.5 μM) for analysis of other BZDs. An aqueous solution of prazepam (12.5 μM) was used as internal standard when analyses were carried out on a DB-1 column. Stock solutions were prepared using methanol as solvent.

Extraction

Internal standard (0.030 ml) and 0.10 ml of 0.1 M phosphate buffer (pH 7.5) were added to 0.50 ml blood and extracted with 0.60 ml *n*-butyl acetate for 2 min on a Multi Tube Vortexer (American Dade, Miami, FL, USA). The samples were centrifuged for 15 min at 1200*g*, and the organic layers were transferred to autosampler vials.

Chromatography

The instruments used were Varian 3400 GCs equipped with Varian 8100 autosamplers and electron-capture detectors (Varian, Palo Alto, CA, USA). Columns: 15 m × 0.32 mm i.d. DB-1701 (14% cyanopropylphenyl) and DB-1 (methylsilicone), 0.25 μm film thickness, obtained from J & W Scientific (Folsom, CA, USA). Injection volume 2 μl, injector temperature 250°C, detector temperature 300°C, helium carrier gas flow 1 ml min^{-1}, nitrogen make-up gas flow 30 ml min^{-1}.

Oven temperature programming DB-1701: 120°C (initial temperature) held for 1 min, 40°C min^{-1} ramp to 230°C, 8°C min^{-1} ramp to 280°C (final temperature), and 9 min hold.

Oven temperature programming DB-1: 120°C (initial temperature) held for 1 min, 30°C min^{-1} ramp to 218°C, 2°C min^{-1} ramp to 230°C, 30°C min^{-1} ramp to 300°C (final temperature), and 1 min hold.

Calculation of results

Drug concentrations were calculated on the basis of the calibration standards. The ratio between peak height of drug and internal standard was used for the calculations.

Table 1
Calibration standards (μM)

Standard no.:	1	2	3	4	5	6	7	8	9
Diazepam	0.25	0.50	1.00	1.50	2.50	5.00	7.50	0	0
Desmethyldiazepam	0.25	0.50	1.00	1.50	2.50	5.00	7.50	0	0
Nitrazepam	0.10	0.20	0.40	0.60	1.00	2.00	3.00	0	0
Flunitrazepam	0.01	0.02	0.04	0.06	0.10	0.20	0.30	0	0
Triazolam	0	0	0	0	0	0	0	0.02	0.20
Flurazepam	0	0	0	0	0	0	0	0.05	1.50
Desalkylflurazepam	0	0	0	0	0	0	0	0.10	3.00
Clonazepam	0	0	0	0	0	0	0	0.10	3.00
Oxazepam	0	0	0	0	0	0	0	0.50	10.00

Conversion: drug concentrations of 1.00 μM correspond to 285 ng ml^{-1} for diazepam, 271 ng ml^{-1} for desmethyldiazepam, 281 ng ml^{-1} for nitrazepam, 313 ng ml^{-1} for flunitrazepam, 342 ng ml^{-1} for triazolam, 388 ng ml^{-1} for flurazepam, 289 ng ml^{-1} for desalkylflurazepam, 314 ng ml^{-1} for clonazepam, and 287 ng ml^{-1} for oxazepam.

Results and Discussion

Chromatograms of a spiked blood sample and of a sample from a driver suspected of drug abuse are presented in Fig. 1. In drug-free blood samples no interfering peaks were found (results not shown).

Retention times

Relative retention times for the BZDs screened for, and for some additional BZDs are presented in Table 2. All BZDs studied were separated when analysed on a DB-1701 capillary column. However, when analysed on a DB-1 capillary column desalkylflurazepam and diazepam were not separated.

Because of a better separation of BZDs on a DB-1701, this column is recommended when screening for BZDs. In cases where confirmation analyses are required and both desalkyl-

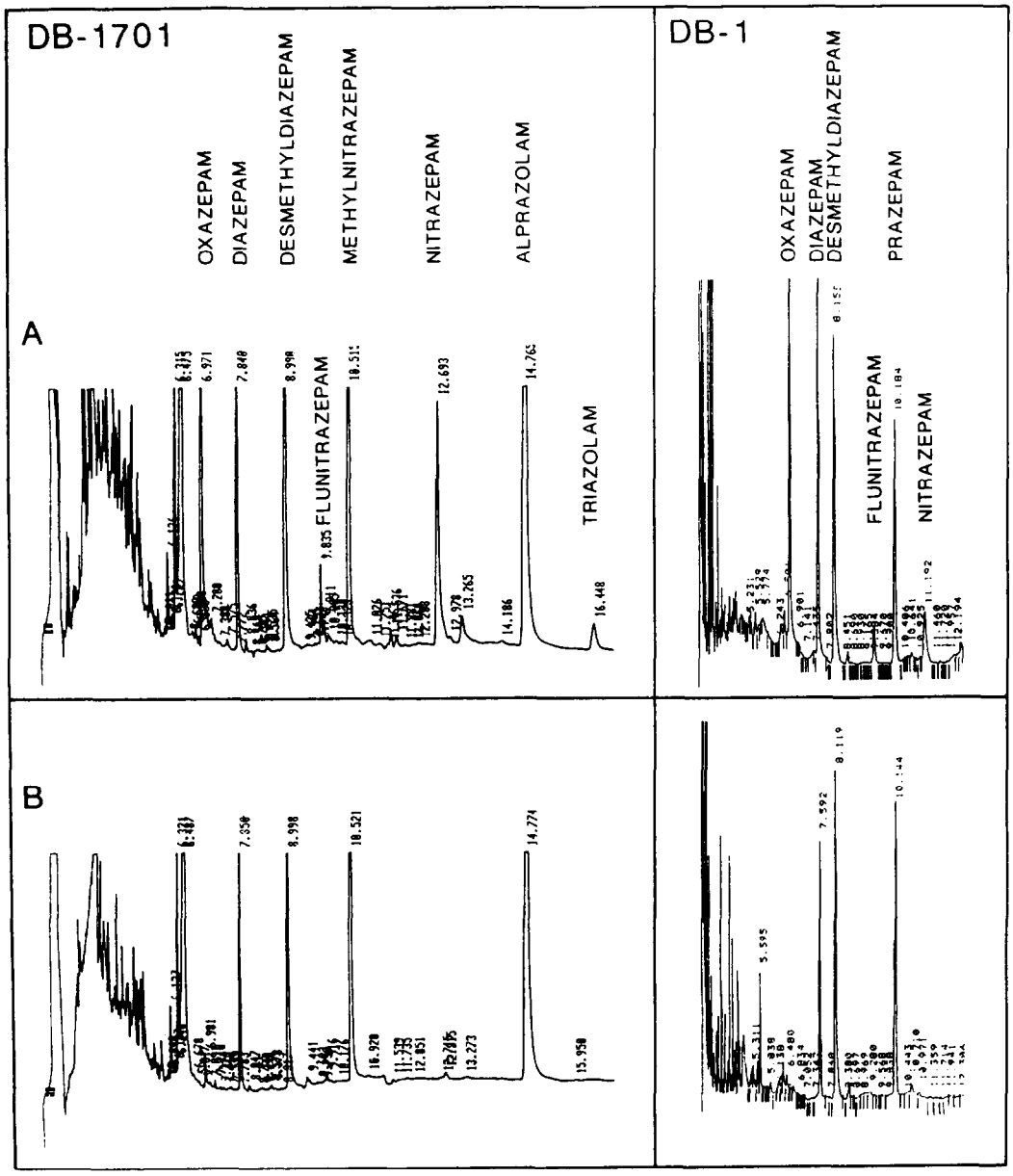

Figure 1
Chromatograms from (A) a spiked blood sample containing 2.00 μM oxazepam, 1.00 μM diazepam, 1.00 μM desmethyldiazepam, 0.04 μM flunitrazepam, 0.40 μM nitrazepam and 0.04 μM triazolam; and (B) a blood sample from a driver suspected of drug abuse estimated to contain 0.4 μM diazepam and 0.7 μM desmethyldiazepam. Internal standards: methylnitrazepam and alprazolam when analysed on DB-1701, and prazepam when analysed on DB-1 capillary columns, concentrations corresponding to 0.75 μM in blood.

Table 2
Relative retention times for common benzodiazepines

	DB-1701	DB-1
Medazepam	0.56	0.70
Oxazepam	0.66	0.68
Diazepam	0.75	0.77
Desalkylflurazepam	0.82	0.77
Midazolam	0.85	0.96
Desmethyldiazepam	0.86	0.83
Prazepam	0.88	1.04
Flunitrazepam	0.94	0.97
Flurazepam	0.95	1.14
Bromazepam	0.96	0.96
Methylnitrazepam	1.00	1.00
Chlordiazepoxide	1.15	1.15
Nitrazepam	1.21	1.11
Clonazepam	1.33	1.16
Estazolam	1.35	1.19
Alprazolam	1.40	1.22
Triazolam	1.56	1.26

Internal standard: methylnitrazepam.

flurazepam and diazepam are found when using a DB-1701 column, a DB-1 column cannot be used for this purpose. In such cases other methods should be used for the confirmation.

Specificity

The electron-capture detector is highly sensitive to molecules such as alkyl halides, conjugated carbonyls and nitrates, and less sensitive to ketones, amines, alcohols and ethers [17]. Several drugs thus can be detected in addition to BZDs. Drugs that might be detected with this type of detector were selected, and fresh solutions in *n*-butyl acetate analysed on the DB-1701 column, 50–100 pmol was injected, corresponding to suprathera-peutic levels of the drugs. The following drugs were tested: brompheniramine, carbamaze-pine, clemastine, clomipramine, clopenthixol, chlormezanone, chloroquine, chlorpromazine, chlorprothixen, chlorzoxazone, disopyramide, dixyrazine, fluphenazine, flupentixol, griseo-fulvin, haloperidol, indomethacin, isosorbide dinitrate, isosorbide mononitrate, mecli-zine, methotrimeprazine, metochlopramide, nifedipin, nitrofurantoin, papaverine, per-phenazine, phenobarbital, prochlorperazine, quinidine, scopolamine, sulindac, trifluo-perazine and verapamil.

Most of the drugs gave very small peaks if detected at all, and as a consequence would not be expected to interfere with the analysis of BZDs. Some drugs gave fairly large peaks with retention times less than those of BZDs (chlor-mezanone and chlorzoxazone). However, nifedipin, griseofulvin and sulindac gave sig-nificant peaks with retention times between 9 and 13 min. In order to study these drugs in more detail, blood samples were spiked at a concentration of 10 μM. The analytical re-covery was very low for sulindac, and no peaks were detected when the extract was analysed. Nifedipin gave three peaks close to flunitraze-pam, whilst griseofulvin gave one peak close to methylnitrazepam. When analysed on a DB-1 capillary column, nifedipin and griseofulvin were very well separated from the benzodi-azepines tested, and from the internal stan-dard. Thus, neither the presence of nifedipin nor griseofulvin can be misinterpreted as any of the tested benzodiazepines when analysed and confirmed on both DB-1701 and DB-1 capillary columns.

Chlormezanone gave large peaks with rela-tive retention times of 0.72 and 0.59 on DB-1701 and DB-1, respectively, and was well separated from BZDs. Chlormezanone is a commonly used muscle relaxant which also is frequently analysed in forensic toxicology. It seems that the present method can be used for screening and confirmation of chlormezanone in addition to BZDs. This was tested further, and it was found that the detection limit for chlormezanone was 0.053 μM when using a DB-1701 column, which is much lower than therapeutic concentrations, which are usually above 17 μM [16].

Recovery

The recovery of internal standards and BZDs in concentrations corresponding to low and high therapeutic levels was studied. The recoveries were found to range from 79 to 98% (results not shown).

Detection limits

The detection limits for the BZDs studied are presented in Table 3. The detection limit was defined as the drug concentration required to give a peak height equal to the mean plus three SD of the background fluctuations of blanks (as recommended by IUPAC [18]). To estimate the background fluctuations, 10 dif-ferent blank blood samples obtained from a blood bank were analysed.

Calibration curves

For a study of the linearity of the calibration, 10 calibration curves selected at random from a

Table 3
Detection limits for representative benzodiazepines

	DB-1701	DB-1
Diazepam	0.096	0.084
Desmethyldiazepam	0.041	0.160
Nitrazepam	0.082	0.088
Flunitrazepam	0.015	0.008
Triazolam	0.005	n.s.
Flurazepam	0.033	n.s.
Desalkylflurazepam	0.018	n.s.
Clonazepam	0.067	n.s.
Oxazepam	0.058	n.s.

n.s. = not studied.

period of 12 months were examined. Most of the calibration curves were found to be linear for diazepam, desmethyldiazepam, nitrazepam and flunitrazepam (concentration ranges are presented in Table 1). However, in some cases the calibration curves were found to be linear for more limited concentration ranges. This may be due to changes in the columns during use. Therefore, neither a calibration curve nor data on linear correlation coefficients are presented.

Precision and accuracy

Data on analytical precision within series are presented in Table 4. Data on total precision (one aliquot of spiked samples analysed in each series [19]) and accuracy are presented in Table 5. It seemed that the determinations were more precise and accurate when using a DB-1701 column. A DB-1701 column therefore is preferred for quantification, whilst a DB-1 column could be used for confirmation.

Table 4
Precision within series for the gas chromatographic assay of benzodiazepines

	Spiked conc. (μM)	DB-1701 RSD	DB-1701 *n*	DB-1 RSD	DB-1 *n*
Diazepam	0.50	16.7	10	5.5	8
	1.50	7.5	10	5.6	8
	5.00	7.1	10	4.5	8
Desmethyldiazepam	0.50	10.5	10	6.0	8
	1.50	5.3	10	5.1	8
	5.00	2.6	10	4.8	8
Nitrazepam	0.10	10.5	10	8.5	8
	0.40	4.4	10	11.5	8
	1.00	6.8	10	11.2	8
Flunitrazepam	0.040	4.8	10	8.3	8
	0.100	2.4	10	6.8	8

Conclusions

A simple and rapid method for analysis of common benzodiazepines in blood has been developed. A DB-1701 capillary column is found to yield better separations between different benzodiazepines than the DB-1 column and therefore is recommended as the primary method for screening and quantification.

Acknowledgements — The authors wish to thank Anita Brautc, Elin Eliassen, Ester Hansen, Gunnar Kinn and Eva Kvernes for technical assistance.

Table 5
Total precision and accuracy (aliquots of spiked samples analysed on *n* analytical series, one aliquot of each sample in each series)

		Analysed concentrations							
		DB-1701				DB-1			
	Spiked conc. (μM)	Mean	RSD (%)	Error (%)	*n*	Mean	RSD (%)	Error (%)	*n*
Diazepam	0.40	0.42	17.1	+4.8	10	0.42	34.1	+4.8	9
	0.50	0.51	10.0	+2.0	12	0.55	8.2	+10.0	13
	0.50	0.65	11.1	+30.0	10	0.54	8.2	+8.0	10
	1.50	1.51	6.0	+0.7	10	1.60	14.1	+6.7	10
	5.00	4.93	5.4	−1.4	10	5.10	12.9	+2.0	10
Desmethyldiazepam	0.39	0.42	18.2	+7.7	11	0.41	29.4	+5.1	10
	0.50	0.52	8.9	+4.0	10	0.58	10.8	+10.8	12
	0.54	0.56	7.4	+3.7	12	0.55	10.7	+10.0	10
	1.50	1.39	6.2	−7.3	10	1.56	10.5	+4.0	10
	5.00	4.73	7.6	−5.4	10	5.04	8.3	+0.8	10
Nitrazepam	0.28	0.30	11.7	+7.1	5	0.28	13.7	0	7
	0.40	0.41	7.8	+2.5	10	0.40	20.5	0	10
	1.00	1.04	5.4	+4.0	10	0.99	17.7	−1.0	8
Flunitrazepam	0.029	0.029	6.6	+0	12	0.032	20.6	+10.3	12
	0.030	0.031	11.5	+3.3	8	0.033	10.2	+10.0	9
	0.040	0.039	7.7	−2.5	10	0.047	9.4	+17.5	10
	0.100	0.096	4.2	−4.0	10	0.112	9.7	+12.0	10

References

[1] M.J. Ellenhorn and D.G. Barceloux, *Medical Toxicology*, pp. 580–586. Elsevier, New York (1988).
[2] J.H. Woods, J.L. Katz and G. Winger, *Pharmacol. Rev.* **39**, 251–413 (1987).
[3] B.S. Finkle, K.L. McCloskey and L.S. Goodman, *J. Am. Med. Ass.* **242**, 429–434 (1979).
[4] J.C. Garriott and N. Latman, *J. Forensic Sci.* **21**, 398–415 (1976).
[5] S.H. Cosbey, *Forensic Sci. Int.* **32**, 245–258 (1986).
[6] H.W. Peel and W.K. Jeffrey, *Can. Soc. Forensic Sci. J.* **23**, 75–79 (1990).
[7] A. Bjørneboe, G.E.A. Bjørneboe, H. Gjerde, A. Bugge, C.A. Drevon and J. Mørland, *Forensic Sci. Int.* **33**, 243–251 (1987).
[8] C. Suitheimer and I. Sunshine, in *Methodology for Analytical Toxicology* (I. Sunshine and P.I. Jatlow, Eds), Vol. 2, pp. 25–30. CRC Press, Boca Raton, FL (1982).
[9] C.E. Lau, S. Dolan and M. Tang, *J. Chromatogr.* **416**, 212–218 (1987).
[10] J.B.F. Lloyd and D.A. Parry, *J. Chromatogr.* **449**, 281–297 (1988).
[11] D.J. Greenblatt, in *Methodology for Analytical Toxicology* (I. Sunshine and P.I. Jatlow, Eds), Vol. 2, pp. 19–24. CRC Press, Boca Raton, FL (1982).
[12] L. Ulrich and F. Sager, *Beitr. Gericht. Med.* **46**, 135–141 (1988).
[13] H. Bun, C. Dechamp, P. Coassolo and J.P. Cano, *Anal. Lett.* **22**, 897–913 (1989).
[14] P. Lillsunde and T. Seppälä, *J. Chromatogr.* **533**, 97–110 (1990).
[15] C. Drouet-Coassolo, C. Aubert, P. Coassolo and J.P. Cano, *J. Chromatogr.* **487**, 295–311 (1989).
[16] A.H. Stead and A.C. Moffat, *Human Toxicol.* **3**, 437–464 (1983).
[17] E.D. Pellizzari, *J. Chromatogr.* **98**, 323–361 (1974).
[18] Analytical Methods Committee, *Analyst* **112**, 199–204 (1987).
[19] M.J. Bookbinder and K.J. Panosian, *Clin. Chem.* **32**, 1734–1737 (1986).

[Received for review 21 October 1991; revised manuscript received 14 January 1992]