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Short communication

Simultaneous determination of twelve benzodiazepines in human serum using a new reversed-phase chromatographic column on a 2-μm porous microspherical silica gel

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Abstract

A high-performance liquid chromatographic method has been developed for the simultaneous analysis of twelve frequently used benzodiazepines (BZPs) (bromazepam, clonazepam, chlordiazepoxide, estazolam, etizolam, flutazoram, haloxazolam, lorazepam, nitrazepam, oxazolam, triazolam and diazepam, internal standard) by using commercially available 2 or 5 μm particle size reversed-phase columns and a microflow cell-equipped ultraviolet detector. The separation was achieved using a C_{18} reversed-phase column (condition 1: 100×4.6 mm I.D., particle size 2 μm, TSK gel Super-ODS; condition 2: 100×4.6 mm I.D., particle size 5 μm, Hypersil ODS-C_{18}). The mobile phase was composed of methanol–5 m*M* NaH_2PO_4 (pH 6) (45:55, v/v), and the flow-rate was 0.65 ml/min (conditions 1 and 2). The absorbance of the eluent was monitored at 254 nm. Retention times under condition 1 were shorter than those of condition 2. When the twelve benzodiazepines were determined, sensitivity and limits of quantification were about four to ten times better under condition 1 than under condition 2. The rate of recovery and linearity in condition 1 were approximately the same as those in condition 2. These results show that a new ODS filler with a particle size of 2 μm was more sensitive, provided better separation and was more rapid than that with conventional ODS filler.

Keywords: Benzodiazepines; Bromazepam; Clonazepam; Chlordiazepoxide; Estazolam; Etizolam; Flutazoram; Haloxazolam; Lorazepam; Nitrazepam; Oxazolam; Triazolam; Diazepam

1. Introduction

Benzodiazepine drugs (BZPs) have anticonvulsant, hypnotic, anxiolytic and muscle relaxant actions and are frequently encountered in emergency toxicology screening, drug-of-abuse testing and in forensic medicine examinations [1]. Several high-performance liquid chromatographic (HPLC) methods have already been reported for the determination of BZPs and/or their major metabolites [2–8]. Recently, gradient analysis [7], photodiode array detection [5,6,8] or reductive electrochemical detection analysis [4] of BZPs was developed. The number of BZPs and related drugs assayed ranged from eight to thirty-three [2–8].

Recently, a new reversed-phase chromatographic column, TSK gel Super-ODS, based on 2 μm silica gel, became commercially available from Toshoh (Tokyo, Japan) [9]. This ODS column achieves better resolution and faster separation than that of

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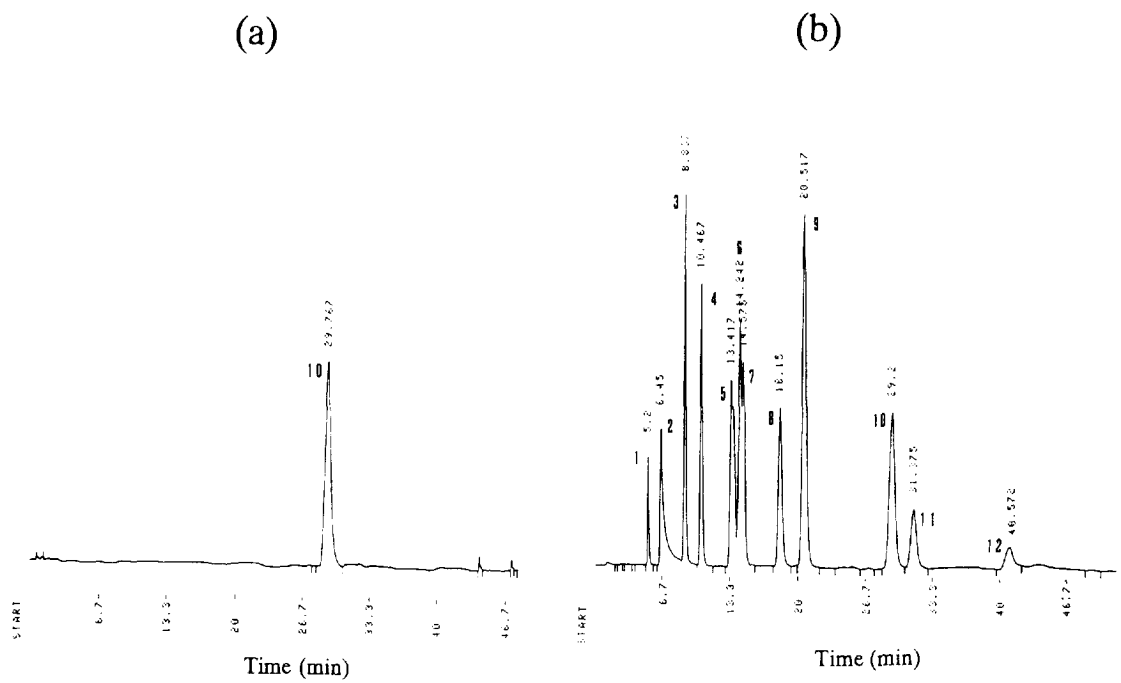

Fig. 1. Chromatograms of twelve benzodiazepines in human sera. Condition 1: column, 100×4.6 mm I.D., particle size 2 μm, TSK gel Super-ODS; mobile phase, acetonitrile–0.5 m*M* NaH_2PO_4 (pH 6) (45:55, v/v); flow-rate, 0.65 ml/min; detection wavelength, 254 nm. (a) Pooled blank serum; (b) drugs added to the drug-free serum. The concentrations of the twelve benzodiazepines are 0.1 μg/ml. 1=clonazepam; 2=bromazepam; 3=nitrazepam; 4=estazolam; 5=triazolam; 6=oxazolam; 7=lorazepam; 8=etizolam; 9=chlordiazepoxide; 10=diazepam (internal standard); 11=flutazoram; 12=haloxazolam.

conventional ODS columns (particle size 5 μm). In this paper, we report the simultaneous determination of twelve BZPs in human serum using this reversed-phase column in comparison with the conventional ODS column.

2. Experimental

2.1. Reagents and materials

Chlordiazepoxide, estazolam and diazepam were a gift from Takeda (Osaka, Japan). Bromazepam was a gift from Kodama (Tokyo, Japan). Clonazepam was a gift from Sumitomo (Osaka, Japan). Etizolam was a gift from Yoshitomi (Osaka, Japan). Flutazoram, haloxazolam and oxazolam were a gift from Sankyou (Tokyo, Japan). Lorazepam was a gift from Yamanouti (Tokyo, Japan). Nitrazepam was a gift from Shionogi (Osaka, Japan). Triazolam was a gift from Nihon Upjohn (Tokyo, Japan). The mobile phase was prepared by mixing deionized water obtained with a Milli-Q system (Millipore, Bedford, MA, USA) and an HPLC-grade organic solvent. Phosphate buffer was made from NaH_2PO_4 (adjusted pH 9 with 1 *M* NaOH).

2.2. Extraction procedure

We added 20 μl of internal standard (diazepam, 20 μg/ml), 200 μl of 1 *M* potassium carbonate and 3 ml of chloroform to 0.5 ml of serum (or standard aqueous solution) to 15-ml teflon-lined screw-capped culture tubes. After mixing for 2 min, the tubes were centrifuged at 1200 *g* for 5 min, and the aqueous phase was removed by aspiration. The organic phase was transferred to a clean conical tube and evaporated in a water bath at about 40°C under a gentle

Table 1
Retention times of eleven benzodiazepines in human sera by the present HPLC method with conditions 1 and 2

Drug	Retention time (min)	
	Condition 1	Condition 2
1. Clonazepam	5.3	18.2
2. Bromazepam	6.6*	25.5*
3. Nitrazepam	9.1	34.2
4. Estazolam	10.7	41.3
5. Triazolam	13.7	43.5
6. Oxazolam	14.6	46.9
7. Lorazepam	15.0	50.1
8. Etizolam	18.4	67.1
9. Chlordiazepoxide	21.0	73.1
10. Diazepam	29.8	77.5
11. Flutazoram	32.2	97.4
12. Haloxazolam	41.5	100.6

Condition 1: Column, 100×4.6 mm I.D., particle size 2 μm (TSK-gel Super ODS). Condition 2: Column, 100×4.6 mm I.D., particle size 5 μm (Hypersil ODS-C_{18}). Diazepam was added as the internal standard. Mobile phase, acetonitrile–5 m*M* NaH_2PO_4 (pH 6) (45:55, v/v); flow-rate, 0.65 ml/min; detection wavelength, 254 nm. All instruments and the column were operated at ambient laboratory temperature (ca. 23°C).
*Tailing peak.

stream of nitrogen. The residue was dissolved in 100 μl of mobile phase, and 20 μl of the solution were injected into the HPLC apparatus.

2.3. Standard solutions and calibration

A standard stock solution, containing twelve BZPs, was prepared at a concentration of 1 mg/ml of each compound in methanol, and it remained stable for at least three months at −20°C. Serum standards were prepared at concentrations of 0.05, 0.5, 1 and 5 μg/ml of each compound by diluting the appropriate aliquots of the stock solution with drug-free serum. The calibration curve was obtained by linear regression of the peak-height ratio.

2.4. Chromatography

The HPLC consisted of a pump (Model CCPS, Tosho) and a variable-wavelength UV detector (Model UV-8000, Tosho), equipped with a 2-μl microflow cell (condition 1) or a 10-μl microflow cell (condition 2) without a heat-sink coil. The separation was achieved using a C_{18} reversed-phase column (condition 1: 100×4.6 mm I.D., particle size 2 μm, TSK gel Super-ODS; condition 2: 100×4.6 mm I.D., particle size 5 μm, Hypersil ODS-C_{18}, Yokogawa, Tokyo, Japan). The mobile phase was composed of acetonitrile–5 m*M* NaH_2PO_4 (pH 6) (45:55, v/v) and the flow-rate was 0.65 ml/min (for conditions 1 and 2). The absorbance of the eluent was monitored at 254 nm. The procedure was performed at ambient laboratory temperature (ca. 23°C).

2.5. Accuracy and recovery

The accuracy and the recovery were calculated by comparing the peak heights of twelve BZPs (0.05, 0.1, 0.5, 1.0 and 5.0 μg/ml) in spiked samples after extraction from serum, to the peak heights of a series of unextracted reference standards.

3. Results and discussion

3.1. Retention time

Fig. 1 shows chromatograms of the twelve BZPs separated using condition 1. Eleven BZPs and the internal standard were well separated. Retention times under condition 1 were shorter than those of condition 2 (Table 1). Under the described optimized chromatographic conditions, they all eluted within 45 min. The reproducibility of the retention time in the analysis of blood samples was better. Cloxazolam [10], mexazolam and oxazepam [11] were not detected with this method, and yet the retention time of alprazolam and triazolam was very similar. These results show that retention times of BZPs mainly depend on the particle size of the reversed-phase column.

No interfering peaks appeared when the following drugs were added to serum; barbital, hexobarbital, pentobarbital, trimethadione, ethosuximide, primidone, phenobarbital, carbamazepine and phenytoin.

3.2. Limits of quantification

The limit of quantification with condition 1 is the lowest concentration on the standard curve that can

Table 2
Precision study of eleven benzodiazepines in human sera by the present HPLC method with condition 1

Drug	Added (μg/ml)	Mean (μg/ml)	Between-day C.V. (%)	Within-day C.V. (%)
1. Clonazepam	0.005	0.0051	6.3	5.6
	0.05	0.051	4.6	4.2
	0.1	0.099	3.8	3.1
2. Bromazepam	0.005	0.0049	6.1	3.3
	0.05	0.048	3.8	3.1
	0.1	0.10	2.9	2.5
3. Nitrazepam	0.001	0.0009	6.9	2.1
	0.05	0.049	4.9	2.7
	0.1	0.11	3.6	2.4
4. Estazolam	0.005	0.0049	6.3	4.2
	0.05	0.051	4.9	3.3
	0.1	0.11	4.6	1.9
5. Triazolam	0.005	0.0048	6.8	2.2
	0.05	0.049	3.2	2.7
	0.1	0.10	4.8	1.8
6. Oxazolam	0.005	0.0049	7.2	1.8
	0.05	0.052	5.1	2.2
	0.1	0.012	4.7	2.6
7. Lorazepam	0.005	0.0049	7.2	1.8
	0.05	0.053	5.6	2.2
	0.1	0.09	4.2	1.7
8. Etizolam	0.005	0.0048	5.2	2.3
	0.05	0.049	4.1	2.7
	0.1	0.098	4.5	2.5
9. Chlordiazepoxide	0.001	0.0009	5.2	2.3
	0.05	0.051	3.6	1.7
	0.1	0.099	4.5	2.3
11. Flutazoram	0.01	0.01	5.5	3.2
	0.05	0.051	3.6	2.4
	0.1	0.098	4.2	2.3
12. Haloxazolam	0.05	0.051	3.6	3.8
	0.1	0.11	4.2	2.3
	0.5	0.51	4.1	2.2

$n=10$. Condition 1: column, 100×4.6 mm I.D., particle size 2 μm (TSK gel Super-ODS); mobile phase, acetonitrile–0.5 m*M* NaH_2PO_4 (pH 6) (45:55, v/v); flow-rate, 0.65 ml/min; detection wavelength, 254 nm. All instruments and the column were operated at ambient laboratory temperature (ca. 23°C). Diazepam was added as the internal standard.

be measured with acceptable accuracy (coefficient of variation (C.V.) <8%). The lower practical limit of quantification was 0.001 μg/ml for chlordiazepoxide and nitrazepam, 0.005 μg/ml for clonazepam, bromazepam, estazolam, triazolam, oxazolam, lorazepam and etizolam, 0.01 μg/ml for flutazoram and 0.05 μg/ml for haloxazolam (Table 2). The sensitivity of eleven BZPs were about five times better under condition 1 than condition 2 (data not shown). This method realized approximately two to ten times higher sensitivity compared with traditional measurement methods that have been reported [4–6,8]. All of these quantification limits are adequate for forensic and clinical analyses [1,6].

3.3. Precision and accuracy

The precision and accuracy obtained using condition 1 is also shown in Table 2. Within-day reproducibility was assessed using ten samples at three different concentrations that were analyzed on the same day. The C.V.s were from 1.7 to 5.6%.

Table 3
Rate of eleven benzodiazepines added to drug-free sera by the present HPLC method with condition 1

Drug	Added (μg/ml)	Mean recovery (%)	C.V. (%)
1. Clonazepam	0.005	96	6.2
	0.1	98	3.3
2. Bromazepam	0.005	101	6.6
	0.1	95	5.3
3. Nitrazepam	0.005	99	2.6
	0.1	95	2.7
4. Estazolam	0.005	95	4.1
	0.1	97	3.2
5. Triazolam	0.005	98	5.1
	0.1	101	3.8
6. Oxazolam	0.005	97	6.1
	0.1	98	4.2
7. Lorazepam	0.005	102	5.7
	0.1	96	3.6
8. Etizolam	0.005	96	4.4
	0.1	98	4.3
9. Chlordiazepoxide	0.001	97	3.8
	0.1	98	3.7
11. Flutazoram	0.1	97	3.8
	1	98	4.2
12. Haloxazolam	0.1	96	4.7
	1	101	3.7

$n=10$. Recovery rate=(actual level/expected level)×100.
Condition 1: column, 100×4.6 mm I.D., particle size, 2 μm (TSK gel Super-ODS); mobile phase, acetonitrile–0.5 m*M* NaH_2PO_4 (pH 6) (45:55, v/v); flow-rate, 0.65 ml/min; detection wavelength, 254 nm. All instruments and the column were operated at ambient laboratory temperature (ca. 23°C). Diazepam was added as the internal standard.

Between-day reproducibility was determined ten times with three different quality control samples, within two weeks. The C.V.s were from 3.6 to 7.2%.

3.4. Recovery

Four liquid–liquid extraction solvents were investigated, including diethyl ether [12], hexane–ethyl acetate [8], chloroform–isopropanol [13] and chloroform. Chloroform was chosen as the extraction solvent because it provided >95% absolute recoveries for BZPs.

To twelve drug-free serum samples were added 0.005 and 0.1 μg/ml of each drug, except for flutazoram and haloxazolam, where 0.1 and 1 μg/ml were added, respectively. Relative recovery was calculated by the values obtained from drug-supplemented serum and the added concentration. The recovery rate ranged from >95% in all drugs (Table 3). The C.V.s were 2.7 to 6.6%. The rates of recovery in condition 1 were approximately the same as those in condition 2 (data not shown).

3.5. Linearity

The calibration curves (the ratio between the peak-height of the drugs analysed and that of the I.S. vs. the amount of each drug) showed linearity in the concentration range of 0.001–0.1 μg/ml of serum, except for flutazoram (0.01–0.1 μg/ml) and for haloxazolam (0.05–0.5 μg/ml). The equations and r values for the curves were $y=0.81x-0.09$, $r=0.998$ for clonazepam; $y=0.83x-0.2$, $r=0.997$ for bromazepam; $y=1.79x-0.68$, $r=0.999$ for nitrazepam; $y=1.21x-0.34$, $r=0.998$ for estazolam; $y=0.95x-0.15$, $r=0.999$ for triazolam; $y=0.98x-0.49$, $r=0.998$ for oxazolam; $y=0.97x-0.46$, $r=0.997$ for lorazepam; $y=0.88x-0.2$, $r=0.999$ for etizolam; $y=2.15x+0.37$, $r=0.997$ for chlordiazepoxide; $y=0.33x-0.08$, $r=0.999$ for fulutazoram; $y=0.16x-0.05$, $r=0.998$ for haloxazolam.

The linearity obtained using condition 1 was approximately the same as that found using condition 2 (data not shown).

A filler with a particle size of 2 μm (TSK gel Super-ODS) and with a pore volume and specific surface that is about one third of that of the conventional filler has recently been developed [9]. This ODS filler has the following advantages: (1) by using this filler, more rapid determination can be expected at room temperature and even without gradient elution, compared with the conventional method; (2) the amount of organic solvent required is smaller and (3) more sensitive determination is possible.

The detection and determination of BZPs with a new ODS filler with a particle size of 2 μm was more sensitive, provided better separation and was more rapid than that with the conventional ODS filler. The rapid turn-around time and accuracy of this method make it suitable for emergency toxicology, forensic toxicology and clinical medicine, allowing detection, confirmation and quantification of many BZPs quickly with a single method.

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