Original article

DETERMINATION OF BENZODIAZEPINE IN SERUM BY THE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC METHOD WITH SOLID PHASE EXTRACTION

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Abstract

Benzodiazepines are among the most prescribed drugs for the treatment of a wide spectrum of clinical disorders. They have been used as anticonvulsants, anxiolytics, hypnotics or muscle relaxants with different durations of action. In this paper, the determination of six frequently used benzodiazepines and metabolites: flunitrazepam, oxazepam, alprazolam, chlordiazepoxide, diazepam and desmethyldiazepam in serum was developed by high-performance liquid chromatography (HPLC) with solid phase extraction (SPE). Quantification was performed with gradient elution of a C8 reversed-phase column, with an alkaline water-acetonitrile-methanol eluent, free of buffer salts. Carbamazepine was detected as an internal standard by ultraviolet absorbance at 239 nm. The determination of flunitrazepam, oxazepam, alprazolam, chlordiazepoxide, desmethyldiazepam and diazepam was possible in a concentration range of 250-4,000 ng/mL, and the mean recovery by this analysis was 91, 91, 93, 84, 110 and 92%, respectively. This method allows rapid detection, a purity check, identification, and quantitation of the eluting peaks. Sensitivity and specificity data are acceptable. The validated procedure has been applied routinely in forensic toxicological analysis. Chiang Mai Med J 2007;46(1):23-30.

Keywords: benzodiazepine, solid phase extraction, high-performance liquid chromatography

Benzodiazepine (BZP) drugs are widely prescribed for their anxiolytic, hypnotic, anticonvulsive and muscle relaxing properties; they are not only important in treating a variety of medical disorders, but also abused by some people. (1-3) Thus, extraction and identification of benzodiazepines in serum is very important for forensic and clinical toxicology. However, an analysis of benzodiazepines in body fluids is quite complex because of the diversity of those available on the market and the fact that each drug has a particular therapeutic and toxic range.
Several analytical techniques for the isolation and quantitation of benzodiazepines in biosamples have already been published. Both thin-layer chromatography (TLC) and the immunoassay approach remain useful for a first rapid screening. However, both techniques have a lack of high specificity. In the case of immuno-analysis, they could not discriminate between a parent drug and metabolites. On the other hand, gas chromatography with flame-ionization (GC/FID) or mass-spectrometric detection (GC/MS) is much more sensitive and specific, but sample derivatization and complex equipment are needed. These reasons have resulted in an increasing popularity of high-performance liquid chromatography (HPLC) for screening and quantitation of benzodiazepines in biosamples. However, several extraction methods for benzodiazepines with liquid-liquid extraction were used for serum and whole blood sample preparation. These methods were not satisfactory because they were too tedious and time consuming.

In this study, we described a rapid and simple solid-phase extraction method and developed an HPLC procedure based on gradient elution of a reversed-phase C8 column with a salt-free eluent. The effluent was monitored by photodiode array detection, with multi-wavelength allowing for identification and quantitation of six different benzodiazepines and their metabolites in postmortem serum by UV detection.

Materials and methods

Apparatus

A high-pressure gradient system was used, consisting of a high-performance liquid chromatography 9012Q Pump Solvent Delivery System, Polychrom 9065 Diod Array Detector and ProStar 310 UV/Vis Detector, a Rheodyne Injector equipped with a 20 mL loop, and star chromatography workstation (Varian Chromatography system, USA). The solid phase extraction system consisted of the Baker-10 SPE™ system, JT Baker, Phillipsburg, NJ., Aspirator A-3S, EYELA Tokyo, Japan., and Bond Elut® HF bond Elut- C18, 500 mg 3 mL, Varian, USA. The HPLC column was Luna C8 (5 mm 100 A 125×4.6 mm.) from Phenomenex®, USA, and the security guard holder and guard cartridge C8 (Octyl,MOS: 4 mm Lx3.0 mm ID) was also from Phenomenex.

Solvents and reagents

Carbamazepine, flunitrazepam, oxazepam, alprazolam, chlordiazepoxide HCl, desmethyldiazepam, diazepam purity 99% and isopropylamine (99%) were purchased from Sigma USA. Methanol and acetonitrile HPLC grade came from Fisher Chemical UK., potassium dihydrogen phosphate was from Fluka Switzerland., and potassium hydroxide pellets and ammonia solution 25% were from Merck, Germany.

Stock solution

All benzodiazepine: flunitrazepam (FNZ), oxazepam (OZP), alprazolam (AZL), chlordiazepoxide (CDZ), desmethyldiazepam (DDZP) and diazepam (DZP) and carbamazepine stock solutions were prepared in methanol (1 mg/mL) and stored at -20°C. They were found to be stable for several months. The working solution was prepared at the appropriate dilution of the stock solution, with a methanol: ultrapure water ratio of 1:1 (v/v).

Chromatographic conditions

The gradient elution, consisting of water containing 0.125% isopropylamine (v/v) (A)
acetonitrile (B) and methanol (C), was applied with the following profile: 0 to 23 min, from 45 (A): 5 (B) : 50 (C) to 20 (A) : 12 (B) : 68 (C) and 23 to 25 min was 20 (A) : 12 (B) : 68 (C).

A flow rate of 0.7 mL/min signals was monitored at 230, 235 and 239 nm, consecutively. The total run time was 25 min. After each run, the system was allowed to equilibrate on the initial solvent conditions for 5 min before injection of the next sample.

Subjects

This study was approved by the Ethical Conduct of Research Involving Humans Committee of the Faculty of Medicine, Chiang Mai University. A serum blank was prepared from pooled serum of twenty healthy volunteers, who had not consumed alcohol or other drugs for 1 month.

Whole blood samples were collected from twenty-five autopsy cases, in which serum screening for benzodiazepine was positive, at the Department of Forensic Medicine, Faculty of Medicine, Chiang Mai University.

The serum samples were stored at -20°C until analyzed. Benzodiazepine and their metabolites were stable for at least 12 months. Samples preparation

After 100 mL of 10 mg/mL carbamazepine in methanol : ultrapure water (1:1, v/v) was added to 1 mL of serum as an internal standard, the samples were diluted with 1 mL of 0.2 M phosphate buffer pH 6.0, and the solution was briefly mixed. The mixture was applied to a bond Elut- C18 cartridge that had previously been activated with 3 mL of MeOH and 3 mL of 0.2 M phosphate buffer pH 6.0. The cartridge was then washed with 3 mL of 50 mL/L of methanol in 0.2 M phosphate buffer pH 6.0, and air vacuumed for 10 seconds. The desired fraction was eluted with 1.2 mL of methanol : 10%NH₃ solution (5 : 1, v/v). The eluate was evaporated to dryness under N₂ gas (99.99%). The residue was dissolved in 200 mL of methanol : ultrapure water (1 : 1, v/v). The samples were injected into the HPLC apparatus.

Method evaluation

Calibration and linearity

Calibration samples were prepared in blank serum by adding the appropriate amount of respective benzodiazepines (250-4,000 ng/mL). Carbamazepine (100 mL of a 10 mg/mL) was used as an internal standard for the different samples. The calibration samples were processed in the same way as the unknown samples. Calibration curves were obtained by plotting the peak area ratio of the analyses to that of the internal standard against the concentration of the respective benzodiazepines.

Extraction recovery

Extraction recovery was evaluated by comparing the peak area of each benzodiazepine in spiked serum with that obtained after injection of a known amount of a standard in mobile phase.

Precision

Within run and between run coefficients of variation were determined by replicate analysis of an aliquot of a sample, in either the same day or on separate days.

Assay detection limit

The limit of detection (LOD) was estimated from extracted serum samples spiked with decreasing concentrations of the benzodiazepines, where the response was equivalent to three times the baseline noise.
The limit of quantification (LOQ) was obtained by the same procedure used for LOD, but estimated as ten times the signal to noise ratio.

**Results and discussion**

The retention time of internal standard and benzodiazepines: flunitrazepam (FNZ), oxazepam (OZP), alprazolam (AZL), chlordiazepoxide (CDZ), desmethyldiazepam (DDZP) and diazepam (DZP) were 7.6, 8.7, 9.1, 10.6, 11.9, 12.7 and 14.3 min, respectively. The chromatographic run time selected was 20 min. Representative chromatograms are shown in Fig. 1. In all drug-free serum samples, only internal standard peaks were found, and endogenous peaks were not observed at the retention time of benzodiazepines (Fig. 1A).

The linearity of the extracted FNZ, OZP, AZL, CDZ, DDZP and DZP was determined by spiking blank serum with known amounts.

![Figure 1. Representative chromatograms of benzodiazepines: flunitrazepam (FZP), oxazepam (OZP), alprazolam (AZL), chlordiazepoxide (CDZ), desmethyldiazepam (DDZP) and diazepam (DZP) detected by ultraviolet absorption at 239 nm. (A) Drug-free serum with carbamazepine (I.S.). (B) Drug-free serum with I.S. and 6 standard benzodiazepines (1,000 ng/mL) added. Chromatograms (C) and (D) are serum samples from autopsy cases (C) chlordiazepoxide (CDZ: 17,069 ng/mL) and (D) desmethyldiazepam (DDZP: 2,493 ng/mL) and diazepam (DZP: 1,622 ng/mL).]
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of each analyst, in order to obtain concentrations of 250, 500, 1,000, 2,000 and 4,000 ng/mL. Fig. 2 shows the standard concentration curves which presented analytical procedure that proved to be linear (squared correlation coefficient $r^2 > 0.995$).

The recovery of FNZ, OZP, AZL, CDZ, DDZP and DZP was determined by adding three different concentrations (250, 1,000 and 3,000 ng/mL) to drug-free serum, and the recovery values were 91% (range 89-94%), 91% (range 89-93%), 93% (range 89-97%), 84% (range 81-87%), 110% (range 93-144%) and 92% (range 84-97%), respectively.

The quantitative evaluation was carried out in serum using six benzodiazepines: FNZ, OZP, AZL, CDZ, DDZP and DZP. The instrumental detection limit of these benzodiazepines was 1.25 to 5.0 ng/injection, and the lower limit of quantitation was 150, 83, 286, 340, 213 and 507 ng/mL, respectively. The examined range was 250 to 4,000 ng/mL for all six benzodiazepines.

The coefficient of variation and relative accuracy values of the six benzodiazepines obtained in the within run and between run assays at a concentration of 500 ng/mL are presented in Table 1. The results show that the within run data were accurate for all benzodiazepines within the acceptance interval of $\pm 20\%$ of the nominal concentration at the quantitation limit. The precision values expressed as coefficient of variation were within the excepted limits of 20% at the quantitation limit. The precision data of DZP were over the excepted quantitation limit (22.1%), because the determined concentration was lower than the LOQ and the non-homogenous of the spiking serum. The between run data on the accuracy values of DDZP and DZP were not acceptable (%RA:

![Figure 2](image_url)

**Figure 2.** Standard concentration curves for FNZ, OZP, AZL, CDZ, DDZP and DZP in serum.

FNZ: $y = 1581.6x - 28.219$, OZP: $y = 794.17x - 2.7842$, AZL: $y = 1031.8x + 0.2575$

CDZ: $y = 868.19x - 5.0003$, DDZP: $y = 662.08x - 70.542$, DZP: $y = 721.6x + 27.273$. 

78.4 and 77.0%). This may be due to decreased benzodiazepines measuring at a low concentration.\(^{(6)}\)

Table 2 shows the results of analysis for benzodiazepines in 25 autopsy cases. Benzodiazepines were tested positive. More than one benzodiazepine was detected in two cases, where there was chlordiazepoxide with diazepam, desmethyldiazepam and oxazepam. In this study, the oxazepam level was higher than that in other benzodiazepines in 18 cases (72%). Two cases were found to have alprazolam and chlordiazepoxide with diazepam over a toxic range.

**Table 1.** Precision and accuracy of the HPLC method for six benzodiazepines (500 ng/mL) in serum

<table>
<thead>
<tr>
<th>Compound</th>
<th>within-run ((n=10))</th>
<th>Between-run ((n=11))</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean</td>
<td>SE</td>
</tr>
<tr>
<td>Flunitrazepam</td>
<td>433</td>
<td>20</td>
</tr>
<tr>
<td>Oxazepam</td>
<td>527</td>
<td>24</td>
</tr>
<tr>
<td>Alprazolam</td>
<td>523</td>
<td>41</td>
</tr>
<tr>
<td>Chlordiazepoxide</td>
<td>526</td>
<td>25</td>
</tr>
<tr>
<td>Desmethyldiazepam</td>
<td>580</td>
<td>54</td>
</tr>
<tr>
<td>Diazepam</td>
<td>492</td>
<td>38</td>
</tr>
</tbody>
</table>

**Table 2.** Results of analysis of benzodiazepine in serum

<table>
<thead>
<tr>
<th>Parent benzodiazepine</th>
<th>Concentration in serum (ng/mL)</th>
<th>Therapeutic/Toxic range (ng/mL)(^{(5,7)})</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No. cases*</td>
<td>Mean</td>
</tr>
<tr>
<td>Flunitrazepam</td>
<td>18 (5)</td>
<td>122</td>
</tr>
<tr>
<td>Oxazepam</td>
<td>1 (1)</td>
<td>325</td>
</tr>
<tr>
<td>Alprazolam</td>
<td>3 (2)</td>
<td>8,865</td>
</tr>
<tr>
<td>Chlordiazepoxide</td>
<td>9 (5)</td>
<td>881</td>
</tr>
<tr>
<td>Desmethyldiazepam</td>
<td>8 (4)</td>
<td>1,169</td>
</tr>
</tbody>
</table>

* ( ) No cases concentration > LOQ

**Conclusions**

This high-performance liquid chromatographic procedure was developed for the simultaneous determination and quantification of six benzodiazepines: flunitrazepam, oxazepam, alprazolam, chlordiazepoxide, desmethyldiazepam and diazepam. It appears that the technique is rapid, simple and suitable for routine analysis. The mobile phase contains no buffer. Isopropylamine for the modification of pH can avoid some problems, which often happened in an HPLC assay when the buffer is used. For example, crystal formed in connecting tubing and detector cells, as well as damage to the pump seals.\(^{(5)}\) This solid phase extraction
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procedure provided cleaner extracts and better recovery than traditional liquid-liquid extraction.\(^4\(^,5\(^,8\(^,9\)\)) The solid phase extraction (SPE) method\(^10\) is easy to use and optimized for isolation of a broad range of drugs from serum. The blank extract from serum gave no peaks to interfere with any benzodiazepines and carbamazepine on the chromatogram. The limit of quantitation was lower than the therapeutic range of the benzodiazepines (except for flunitrazepam and alprazolam). Therefore, the method can be used in routine forensic applications. It can also be used to quantitate known benzodiazepines.

Although there have been more than 35 types of benzodiazepines, the protocol developed in this study shows detection of only 6 most commonly used benzodiazepines.\(^1\(^,4\)\) In our pilot study, this protocol could determine other types of benzodiazepines, such as clonazepam, lorazepam, temazepam, triazolam and midazolam at the retention times of 5.5, 8.8, 10.0, 11.2 and 14.2 min, respectively. However, we could not quantify them in this study because of the lack of pure standards.

Acknowledgments

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References

การตรวจวิเคราะห์ยาเบนโซไดอะซีปีนในซีรั่ม ผ่านการสกัดด้วย solid phase extraction โดยวิธี high-performance liquid chromatography


บทคัดย่อ
ปัจจุบันพบว่ายากลุ่มเบนโซไดอะซีปีนถูกนำมาใช้ในทางที่ผิดอย่างแพร่หลาย ยาในกลุ่มนี้มีคุณสมบัติคือลดอาการวิตกกังวลและซึมเศร้า รักษาอาการเกร็งตัวของกล้ามเนื้อ ควบคุมกล้ามเนื้อสั่นจากการชัก การถอนเหล้า และใช้รักษาอาการนอนไม่หลับที่มีสาเหตุจากความวิตกกังวล งานวิจัยนี้ทำการพัฒนาการตรวจวิเคราะห์ยาเบนโซไดอะซีปีนในซีรั่มที่พบใช้อยู่ 6 ชนิด คือ flunitrazepam, oxazepam, alprazolam, chlordiazepoxide, diazepam และ desmethyldiazepam โดยใช้วิธีสกัดด้วย solid phase extraction (SPE) และวิธี high-performance liquid chromatography (HPLC) การตรวจวิเคราะห์ยาเบนโซไดอะซีปีนในซีรั่มพบว่ามีค่าความคลื่นแสง 239 นาโนเมตร โดยอาศัยสีสีตนบรรจุสารชนิด C8 และตัวทำละลายที่ประกอบด้วยสารละลาย acetonitrile, methanol, สารละลายในระบบปรับเปลี่ยนอัตราส่วนตัวทำละลาย ใช้สารทหารเข้มข้นเป็นสารมาตรฐาน 6 ชนิด คือ flunitrazepam, oxazepam, alprazolam, chlordiazepoxide, desmethyl diazepam และ diazepam จะถูกตรวจวิเคราะห์ในระดับ 0.04 mg/ml. สารนี้จะถูกตรวจสอบในระดับ 0.04 mg/ml. ที่ระดับ 0.04 mg/ml. มีค่าเฉลี่ยของการตรวจพบ (recovery) ที่ระดับ 91, 93, 84, 110 และ 92 ตามลำดับ วิธีนี้สามารถตรวจแยกยาเบนโซไดอะซีปีนในซีรั่มได้ชัดเจน มีความไวและความจำเพาะดี สามารถนำไปปรับใช้ในงานประจำของพิษวิทยาได้ เชียงใหม่เวชสาร 2550;46(1):23-30.

คำสำคัญ: กลุ่มยาเบนโซไดอะซีปีน, ซีรั่มเบนโซไดอะซีปีน