Clonazepam serum levels in epileptic patients determined simply and rapidly by high-performance liquid chromatography using a solid-phase extraction column.

Katsushi Furuno*      Yutaka Gomita†
Yasunori Araki‡       Tamotu Fukuda**

*Okayama University,
†Okayama University,
‡Okayama University,
**Ehime University Medical School,
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Abstract

We studied the use of high-performance liquid chromatography (HPLC), using a solid phase extraction column (Bond Elut cartridge column), for the simple, rapid and sensitive determination of serum clonazepam levels in epileptic patients. Extracted aliquots were analyzed by HPLC, using a reverse phase ODS column (mu-Bondapak C18). The analytical mean recovery of clonazepam added to the blank serum averaged 99.9%. The detection limit was as high as approximately 2 ng/ml in the serum. The reproducibilities were 2.3-8.6 CV % in the within-day assay and 6.5 CV % in the between-day assay, indicating that the analysis method was effective in the determination of clonazepam serum levels. Accordingly, we suggest that the present method, using a solid phase extraction column, may be useful for the routine monitoring of clonazepam serum levels in epileptic patients.

KEYWORDS: clonazepam serum levels, epileptic patient, therapeutic drug monitoring, solid-phase extraction, HPLC

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Clonazepam Serum Levels in Epileptic Patients Determined Simply and Rapidly by High-Performance Liquid Chromatography Using a Solid-Phase Extraction Column

Katsushi Furuno, Yutaka Gomita*, Yasunori Araki and Tamotu Fukuda*

Department of Hospital Pharmacy, Okayama University Medical School, Okayama 700, and *Department of Hospital Pharmacy, Ehime University Medical School, Ehime 791-02, Japan

We studied the use of high-performance liquid chromatography (HPLC), using a solid phase extraction column (Bond Elut cartridge column), for the simple, rapid and sensitive determination of serum clonazepam levels in epileptic patients. Extracted aliquots were analyzed by HPLC, using a reverse phase ODS column (μ-Bondapak C18). The analytical mean recovery of clonazepam added to the blank serum averaged 99.9%. The detection limit was as high as approximately 2ng/ml in the serum. The reproducibilities were 2.3–8.6 CV % in the within-day assay and 6.5 CV % in the between-day assay, indicating that the analysis method was effective in the determination of clonazepam serum levels. Accordingly, we suggest that the present method, using a solid phase extraction column, may be useful for the routine monitoring of clonazepam serum levels in epileptic patients.

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Clonazepam (7-nitro-5-(2-chlorophenyl)-1, 3-dihydro-2H-1, 4 benzodiazepine-2-one, CZP), a benzodiazepine, is used clinically as an anticonvulsant (1–3). It is well known that the therapeutic range of this drug in serum levels during chronic dosing is very narrow and that high doses cause severe side effects such as ataxia and sedation (4, 5). Accordingly, monitoring of CZP serum levels is required in epileptic patients, particularly for controlling the development of epileptic seizures during chronic drug administration.

Many reports concerned with methods of determination of serum and plasma CZP level have been published in connection with the therapeutic monitoring of CZP in epileptic patients. In earlier methods, a gas-liquid chromatograph (GLC), equipped with a Ni-63 electron capture detector, was used for the determination of CZP serum or plasma levels (6, 7). However, use of the GLC method required extraction from serum or plasma, i.e., a sample might be prepared by liquid-phase extraction of the drug followed by modification by methylation, trimethylsilylation, or acid hydrolysis. In contrast, high-performance liquid chromatography (HPLC) has become the method of choice because its selectivity is greater than that of the GLC method (8, 9). However, the pretreatment of the serum or plasma in the
HPLC method has also been laborious. The liquid-liquid phase extraction followed by evaporation and reconstitution with a mobile phase solvent required large sample volumes and considerable time (10, 11).

In the present study, the authors investigated a simpler and more rapid method of monitoring CZP levels in patient serum by means of the HPLC method, using a solid-phase extraction technique.

1D-690 5-(o-chlorophenyl)-1-methyl-7-nitro-1, 3-dihydro-2H-1, 4 benzodiazepine-2-one) was used as the internal standard (I.S.).

A standard stock solution of CZP, for obtaining the standard curve, and an I.S. solution of 1D-690 were prepared by dissolving 10 mg of each drug, respectively, in 100 ml of methanol. The standard stock solution was diluted with 0.05 M ammonium phosphate dibasic solution to obtain concentrations of 20, 40, 80 and 160 ng/ml. The I.S. solution was diluted to 160 ng/ml with 0.05 M ammonium phosphate solution.

All the blood samples obtained from epileptic patients were separated into serum and red blood cells by centrifugation at 3,000 rpm for 15 min, and the serum was frozen at −20°C until the CZP assay was carried out.

A Bond Elut C18 (1 ml volume, Cat. No. 607101, Analytichem International, USA), solid-phase extraction (SPE) cartridge containing octadecyl silica, was used to extract the CZP and I.S. from the serum. The SPE column was set on a Vac Elut chamber (Analytichem International, USA), and was washed twice with 1 ml of methanol, followed by two washings with 1 ml of distilled water. One ml of CZP standard solutions (20–160 ng/ml) or 0.2 to 1 ml of patient serum containing CZP, and 1 ml of I.S. solution were then added to the SEP column. After passing 1 ml of distilled water and 1 ml of 20% methanol through the column, the CZP and I.S. retained on the column were eluted with 250 μl of methanol. Forty-μl methanol eluents were injected into the HPLC system. This volume of methanol did not affect the shape of the chromatogram.

A Hitachi model 228 spectrophotometer was used for choosing an absorption wavelength to detect the CZP in the serum. Serum levels of CZP were calculated using a Data Module (Japan Waters, Type 730) after determination using a HPLC instrument (Japan Waters, Pump-Type 510) with a UV detector (Japan Waters, Type 440). A stainless steel column packed with μ-Bondapak C18 (3.9 mm diameter × 300 mm length, and 10 μm particle size, Japan Waters) was maintained at 22 ± 2°C. The sample injection was carried out using an automatic sample processor (Japan Waters, Type WISP 710B) equipped with HPLC instrument. A solution of acetonitrile and distilled water (40 : 60) degassed by ultrasonic vibration was used as the mobile solvent.

The absorption wavelength for the detection of CZP by HPLC was chosen using a methanol solution of 10 μg/ml CZP. The maximum absorption peaks were recorded near 254 and 306 nm in the ultraviolet spectrum. Absorption at 254 nm offered good selectivity for the determination of CZP serum levels, so this absorption wavelength was used for the HPLC detection.

Serum CZP was determined using a mobile solvent with a flow-rate of 1.5 ml/min at room temperature. The absorbance of eluent was detected at 254 nm, and the sensitivity was set at 0.005 a.u.f.s. The standard curve for the determination of the CZP serum level was based on the peak-area ratio of CZP to the I.S.

The chromatographic peaks of CZP and I.S. were coincident with those of authentic standards. After the addition of CZP standards of 20, 40, 80 and 160 ng/ml, each solution was injected into the HPLC instrument. The standard curve for CZP levels was obtained from the peak area ratio of CZP and I.S. on the chromatogram.

For obtaining the recovery rate of CZP, after passing 1 ml of the known CZP serum level at a level of 10.7, 21.5, 32.8, or 50.0 ng/ml, 1 ml of CZP standard solution at a concentration of 20 or 40 ng/ml, and 1 ml of I.S. solution were added to
the SEP column. The recovery rate was calculated from the value measured by HPLC.

To investigate the precision of the present assay, replicate serum samples obtained from epileptic patients to whom CZP had been administered, were measured 10 times in the within-day assay and 7 times in the between-day assay (7 days). For investigating whether it was possible to determine CZP serum levels using a small sample volume, sample solutions of 0.2, 0.4, 0.8, or 1 ml of known CZP serum levels (approximately 30 ng/ml) were tested in triplicate.

As shown in Fig. 1, the retention times of CZP and the I.S. on the chromatogram were 7.0 and 10.6 min, respectively. The peaks corresponding to CZP, the I.S. and other antiepileptics such as phenytoin, phenobarbital, carbamazepine, valproate and their metabolites, were separated well, indicating that interfering endogenous substances were not present. The patient serum CZP level obtained in the present assay was 18.5 ng/ml, which was calculated by CZP peak area/I.S. peak area. An excellent linear correlation was noted in the range of 20 to 160 ng/ml, between the serum level of CZP and the peak-area ratio of CZP and I.S. The coefficient of correlation was \( r = 0.999 \), and the regression equation was \( y = 0.0075x + 0.0028 \). The detection limit calculated with an S/N (signal/noise) ratio of 3 was approximately 2 ng when 1 ml of serum was analyzed. The rates of recovery, calculated by adding CZP at concentrations of 20, 40, 50 or 100 ng/ml to the known CZP serum levels, averaged 99.9 % (from 90.3 to 109.6 %).

To test the precision of the assay of CZP serum levels, the present method was used to perform a within-day assay and a between-day assay. The coefficients of variation (CV) in the unknown serum sample I and II were 2.3 % (114.5 ± 2.6 ng/ml, \( n = 10 \)) and 8.6 % (26.2 ± 2.2, \( n = 10 \)), respectively, in the within-day assay. The CV in the unknown serum sample, III, was 6.5 % (49.6 ± 3.2 ng/ml) in the between-day assay (7 days).

There was an excellent linear correlation between the tested serum volume and the CZP/I.S. peak area ratio (Table 1). The coefficient of correlation was \( r = 0.9983 \) (p < 0.01), and the

Fig. 1  Typical chromatograms for clonazepam (CPZ) and the internal standard (I.S., ID-690). (A) : CZP-free blank serum in an epileptic patient to whom phenytoin, phenobarbital, carbamazepine, and valproate had been administered. (B) : CZP (40 ng/ml) and the I.S. (160 ng/ml) were added to the blank serum. (C) : The serum of a patient to whom CZP had been administered. The CZP level in this serum was 18.5 ng/ml.
Table 1  The determination of clonazepam levels in various volumes of the same serum sample

<table>
<thead>
<tr>
<th>Sample volumes (ml)</th>
<th>n</th>
<th>CZP levels (ng/sample volume)</th>
<th>CZP levels (ng/ml)</th>
<th>CV* (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.2</td>
<td>3</td>
<td>5.8 ± 0.6</td>
<td>29.0 ± 4.0</td>
<td>13.8</td>
</tr>
<tr>
<td>0.4</td>
<td>3</td>
<td>11.4 ± 0.5</td>
<td>28.4 ± 1.3</td>
<td>4.6</td>
</tr>
<tr>
<td>0.8</td>
<td>3</td>
<td>25.9 ± 1.9</td>
<td>32.4 ± 2.3</td>
<td>7.7</td>
</tr>
<tr>
<td>1.0</td>
<td>3</td>
<td>31.0 ± 1.3</td>
<td>31.0 ± 1.3</td>
<td>4.2</td>
</tr>
</tbody>
</table>

Each value indicates the mean ± S.D. There was a good correlation between sample volumes and CZP levels (r = 0.9983, p < 0.001). The regression equation for the data is y = 32.45x − 0.94 (x, sample volume; y, CZP levels in each sample volume). a : Coefficients of variation

regression equation was \( y = 32.45x - 0.94 \). Values of CZP levels, determined using sample volumes of 0.2, 0.4, 0.8, and 1.0 ml, were 29.0, 28.4, 32.4, and 31.0 ng/ml, respectively.

Regarding methods for the determination of CZP in serum or in plasma, liquid-liquid extraction from serum and plasma has already been tested. However, these liquid-liquid extraction methods had a few problems related to the pre-treatment of CZP, e.g., serum and plasma extraction was complicated and there was a further need to evaporate the sample after extraction. Concerning the treatment of serum and plasma sample preparations, a solid-phase extraction method has recently been developed for simply and rapidly monitoring or measuring a number of drugs in biological fluids (11, 12). In the present experiment, solid-phase extractions, using a Bond Elut cartridge mini column containing octadecyl-bonded silica, was used in the pre-treatment step of the HPLC method for determining CZP serum levels. As the results showed, the present method was simpler and more rapid, more selective and more accurate than the liquid-liquid extraction formerly used, indicating its usefulness for determining CZP levels in patient serum.

On the other hand, for clinically monitoring antiepileptic drugs such as phenytoin, phenobarbital, carbamazepine and so on, an enzyme immunoassay is often used, in which the sample volume is less than approximately 50 μl of serum (13, 14). However, sample volumes so far used for HPLC determination of CZP serum levels have been more than approximately 1 ml (8–9). The sample volume is one of the most important factors in clinical drug monitoring. From small infants, in particular, it is not so easy to obtain a large volume of blood. The detection limit for CZP in the present method was approximately 2 ng/ml, and there was a good linear correlation between sample volumes used for the determination and CZP levels in the same serum. It is said that optimal clinical CZP levels for patient serum are 20-80 ng/ml (4–5). Accordingly, if CZP levels are above 10 ng/ml, it is possible to obtain CZP levels using a small sample volume of 0.2 ml. The present method may be useful for the therapeutic drug monitoring of CZP serum levels even in small infants.

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Clonazepam Determination in Serum Levels


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