High-Performance Liquid Chromatographic Assay of Clonazepam in Human Plasma Using a Non-porous Silica Column

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A new HPLC method has been developed for measuring clonazepam (CZP) in plasma, using a reversed-phase non-porous silica column packed with 2 μm particles. CZP in plasma was first purified with a column extraction technique and injected onto a non-porous silica column. The calibration curve was linear from 5—200 ng/ml. The recoveries of CZP added to plasma were more than 94.0%, with a coefficient of variation in the range of 5.1—13.8%. We developed a rapid routine method using a non-porous silica column that was accurate and improved solvent consumption in the measurement of CZP.

Key words clonazepam; non-porous silica column; HPLC; benzodiazepine; column extraction

Clonazepam (CZP, 5-(2-chlorophenyl)-1,3-dihydro-7-nitro-2H-1,4-benzodiazepin-2-one) has anticonvulsant properties.1) The optimal therapeutic range of CZP during chronic treatment has been reported in the range of 15—80 ng/ml.2) The measurement of CZP levels is important in maintaining a therapeutic concentration without side-effects.

Many HPLC methods using a conventional reversed-phase porous silica column for monitoring the plasma concentration of CZP have been reported.3—8) However, these methods have common problems including long analysis time and high solvent usage. Recently, it has been reported that 1.5—1.6 μm particle non-porous silica columns achieve better resolution, and provide superior performance over the 5—10 μm particle porous silica columns in several parameters in the separation of peptides.9—11) HPLC methods using the non-porous silica column were faster and used less solvent in the analysis of several drugs in plasma.12,13) Furthermore, Spell et al. suggested in their preliminary experiments of CZP assay that the non-porous silica column gave a short retention time, which lead to less solvent consumption.14) For that reason we evaluated the possibility of using a reversed-phase non-porous silica column on the HPLC assay of CZP in human plasma. Ultimately, we developed a routine method on the non-porous silica column that was rapid and accurate, and improved solvent consumption in the measurement of CZP.

MATERIALS AND METHODS

Reagents CZP, diazepam, phenobarbital, carbamazepine, nitrazepam, and butyl p-hydroxybenzoate (internal standard, I.S.) of analytical grade were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). The structure of CZP is shown in Fig. 1. 7-Aminoclonazepam was supplied by Dainippon Pharmaceutical Co., Ltd. (Osaka, Japan). Ethosuximide was supplied by Sankyo Co., Ltd. (Tokyo, Japan). Paroxetine was supplied by Glaxo-SmithKline PLC (England, U.K.). Sertraline was supplied by Pfizer, Inc. (Groton, CT, U.S.A.). Milnacipran was supplied by Asahi Kasei Corporation (Tokyo, Japan). Acetonitrile, chloroform and distilled water were of liquid chromatographic grade. Other reagents were of analytical-reagent grade.

Patients’ Samples Patient plasma samples were obtained from “waste” plasma samples remaining after routine therapeutic drug monitoring in Gifu University Hospital. Informed consent was obtained from all of the patients.

Apparatus and Chromatographic Conditions The HPLC system LC-10A (Shimadzu, Kyoto, Japan) consisted of a pump (LC-10), UV detector (SPD-10A V) and autoinjector (SIL-10AXL). Condition 1: reversed-phase non-porous silica column packed with 2 μm particles, Presto FT-C18 (30×4.6 mm I.D., Imtak, Kyoto, Japan), was used. The mobile phase was composed of acetonitrile—1% acetic acid (10:90, v/v). The mobile phase flow-rate was 1.0 ml/min, and separation was performed at 35 °C. Condition 2 (the conventional HPLC method): 5 μm reversed-phase porous silica column packed with 5 μm Cosmosil C18 (250×4.6 mm I.D., Nacalai Tesque, Tokyo, Japan) was used. The mobile phase was acetonitrile—20 mM phosphate buffer (pH 3.5) (40:60, v/v). The mobile phase flow-rate was 1.5 ml/min, and separation was performed at 70 °C. The elute was monitored at 306 nm with a sensitivity of 0.02 a.u.f.s. The chromatographic data were calculated with a Shimadzu CHROMATOPAC C-R7Ae.

Standard Solutions A standard stock solution of CZP was prepared in methanol at 1 mg/ml, stored at −20 °C, and found to be stable for six months. This solution was diluted

Fig. 1. Structure of Clonazepam

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with methanol to obtain concentrations of 5, 10, 50, 100 and 200 ng/ml. A 1 ml volume of each solution was pipetted into a 10 ml test tube, and the methanol was evaporated. The residue of each standard was then dissolved in 1 ml of drug-free plasma, frozen and stored at −20 °C until the assay. CZP in plasma was stable for at least three months at −20 °C. The I.S. solution was prepared at 100 µg/ml in Milli-Q water.

Sample Preparation The E.Xtrelut NT1 (5 ml volume) for a column extraction technique, followed by HPLC, was obtained from Merck (West Point, PA, U.S.A.). Plasma (500 µl), 200 µl of 0.5 M sodium phosphate (pH 9.2) and 100 µl of I.S. solution were applied to the column. CZP and I.S. were eluted with 6 ml of chloroform. This elute was evaporated to dryness. The residue was reconstituted in 100 µl of mobile phase, and filtered with a 0.2 µm membrane filter (Millex-LG, Millipore, Bedford, MA, U.S.A.). Then 50 µl was injected onto the HPLC.

Calibration Curve CZP was dissolved in drug-free plasma in the range of 5—200 ng/ml. The plasma samples were treated according to the above procedure. Peak-area ratios of CZP to I.S. were measured and plotted against the concentration of CZP in plasma.

Accuracy and Recovery The accuracy and recovery were calculated by comparing the concentrations of CZP (10, 50, 100 ng/ml) in spiked samples, after extraction from serum, to the concentrations of a series of unextracted reference standards.

RESULTS

Figure 2 shows representative chromatograms for blank plasma, blank plasma spiked with CZP, and the plasma of a patient receiving CZP. The retention times of CZP and I.S. were 1.8 and 3.3 min, respectively. No interfering peaks of endogenous substances were present. The peaks corresponding to CZP and I.S. were well separated from those of other anticonvulsant drugs such as phenobarbital, phenytoin, nitrazepam, carbamazepine, fluoxetine, diazepam, clobazam and some of their metabolites (Fig. 2B, Table 1). Paroxetine, sertraline, milnacipran, p-hydroxyphenobarbital, ethosuximide, primidone, zonisamide, 7-aminoconlazepam, 7-aminoconlazepam and 7-acetamidenitrazepam were eluted at void volume (0.3 min). N-Desmethylclobazam, however, showed a retention time of 1.8 min, and its presence made the analysis impossible.

A linear correlation was shown between the peak-area ratio of CZP to I.S. and the CZP concentration in plasma over the range of 5—200 ng/ml. The regression equation was

\[ y = 0.025x + 0.045 \]  

where \( y \) is the peak-area ratio of the drug to the I.S., \( x \) is the concentration (ng/ml) of the drug in plasma and \( r \) is the correlation coefficient. The lower limit of determination, calculated with a 3×S/N ratio, was 2.1 ng/ml for CZP.

The precision of the determination of CZP was examined by performing six replicate analyses at each of the three different concentrations of the drug in plasma. Intra-day precision of the procedure was determined by analyzing the plasma with concentrations of 10, 50 and 100 ng/ml CZP. Coefficients of variations (C.V.) ranged from 7.7 to 13.8%. For the inter-day precision, 500 µl of quality control plasma, analyzed in six consecutive runs, gave C.V. values in the range of 5.0—11.9%. A summary of these data is given in Table 2.

The accuracy test for the determination of CZP in plasma was examined by adding a known amount of CZP to blank plasma at 10, 50 and 100 ng/ml. The results are summarized in Table 2. The recoveries for CZP were 94.0—106.6%.

In Fig. 3, we observed a good correlation between the plasma CZP concentrations in fifteen patients receiving CZP using our new HPLC method with a non-porous silica column, and the values obtained with our conventional HPLC method with a porous silica column. For the conventional HPLC method, the retention times of CZP and \( \alpha \)-naphtyl-
amine (I.S.) were 5.7 and 6.3 min, respectively. The regression equation was \( y = 0.959x + 0.206 \) \((r^2 = 0.878)\), where \( y \) is the concentration (ng/ml) obtained by the new HPLC method, \( x \) is the concentration (ng/ml) obtained by the conventional HPLC method, and \( r \) is the correlation coefficient.

**DISCUSSION**

It was reported that 306—313 nm wavelength were used to minimize the absorbance from biological substances and to avoid interference from other drugs.\(^{3,5,8}\) We used 306 nm wavelength to eliminate the interference in the low-nanogram range of CZP therapeutic concentration. The limit of detection was 2.1 ng/ml. This is below the therapeutic range (15—80 ng/ml). The sensitivity of the present method compares favorably with these obtained by other methods.\(^{3—8}\) Replicate analyses indicated low C.V. In the accuracy test, CZP recovery was more than 94.0%. These parameters were satisfactory in the clinical range.

It was reported that the amount of organic modifier needed for a separation was reduced by over 90% on the non-porous silica versus porous silica columns.\(^{12,13}\) The HPLC methods for CZP, using a conventional porous column (250×4.6 mm I.D., 5 \( \mu \)m particle-size), needed large amounts of organic modifier as follows: 40% organic modifier, 1.0 ml/min,\(^{5}\) 35% organic modifier, 3.0 ml/min,\(^{4}\) 25% organic modifier, 2 ml/min,\(^{5}\) 41% organic modifier, 1.2 ml/min,\(^{6}\) 40% organic modifier, 1.3 ml/min,\(^{7}\) 50—70% organic modifier, 1.0 ml/min.\(^{8}\) In our conventional method, CZP was eluted on a porous silica column using a 40% isocratic organic modifier, 1.5 ml/min. In contrast, in our new HPLC method by the non-porous silica column, CZP was eluted at a 1.0 ml/min flow rate, using a 10% organic modifier.

The non-porous silica column packed with 1.5 \( \mu \)m particles (33×4.6 mm I.D.) gave a short retention time of 1.72 min (20% organic modifier, 1 ml/min) for CZP.\(^{13}\) In our method by the non-porous silica column, CZP was eluted up to 1.8 min. In other reports\(^{3—8}\) and with our conventional HPLC method, CZP was eluted between 3.4—6.5 min. CZP was analyzed up to 1.9—3.6 times faster compared to the conventional porous columns.

The above points demonstrate that the present method, using a non-porous silica column, has a significant advantage with respect to reduction of required solvent and analysis time, and may be useful for routine monitoring or pharmacokinetic studies of CZP.

**REFERENCES**