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Short communication

High-performance liquid chromatographic assay of zonisamide in human plasma using a non-porous silica column

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Abstract

A new method for measuring zonisamide (ZNS) in plasma by high-performance liquid chromatography was developed by using a 2-μm reversed-phase non-porous silica column. ZNS in plasma was first purified with a column extraction technique and injected onto the non-porous silica column. Calibration curve was linear over the concentration range of 1–80 μg/ml in plasma. The recoveries of ZNS added to plasma were more than 95.4% with the coefficient of variation less than 9.0%. We developed a rapid routine method using the non-porous silica column that was accurate and improved solvent consumption in the measurement of ZNS.

Keywords: Zonisamide; Non-porous silica column

1. Introduction

Zonisamide (1,2-benzisoxazole3-methanesulfonamide) is used as an anticonvulsant in patients with epileptic disorders [1–4]. The recommended therapeutic range of the serum concentration of this drug is usually attained with plasma concentrations of 17–50 μg/ml [3]. Determination of the zonisamide (ZNS) serum concentration is required in epileptic patients in order to establish a proper concentration of the drug for the inhibition of epileptic seizures and to avoid side-effects.

High-performance liquid chromatographic (HPLC) assay, using a porous silica column, is commonly used for separation and quantification of many drugs, but it has been reported that 1.5–2.0-μm particle non-porous silica columns provide superior performance over the 3.5–10-μm particle porous silica columns in several parameters [5–8]. The method on the non-porous silica column was faster, used less solvent, and provided greater analyte peak area [5–8]. Several HPLC methods using a conventional reversed-phase porous silica column for monitoring the serum concentration of ZNS have been reported [9–13]. However, these methods have common problems of long run time and high solvent consumption. It is very important to reduce both analysis time and solvent costs without compromising quality. For that reason we evaluated the possibility of using a reversed-phase non-porous silica column on the HPLC assay of ZNS. Ultimately, we developed a routine method on the non-porous silica

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column that was rapid and accurate, and improved solvent consumption in the measurement of ZNS.

2. Experimental

2.1. Chemicals

Zonisamide was kindly supplied by Dainippon Seiyaku (Osaka, Japan). The structure is shown in Fig. 1. Phenobarbital, carbamazepine, clonazepam, nitrazepam and *p*-hydroxy methylbenzoate (internal standard, I.S.) of analytical grade were purchased from Wako (Osaka, Japan). Carbamazepine 10,11-epoxide was obtained from Sigma (St. Louis, MO, USA). Phenytoin, 5-(3-hydroxyphenyl)-5-phenylhydantoin and 5-(4-hydroxyphenyl)-5-phenylhydantoin were obtained from Aldrich (Milwaukee, WI, USA). 7-Acetamidonitrazepam and 7-aminonitrazepam were supplied by Shionogi Seiyaku (Osaka, Japan). 7-Aminoclonazepam was supplied by F. Hoffmann-La Roche (Basel, Switzerland). Acetonitrile, chloroform and distilled water were of liquid chromatographic grade. Other reagents were of analytical-reagent grade.

2.2. Apparatus and chromatographic conditions

The HPLC system LC-10A (Shimadzu, Kyoto, Japan) consisted of a pump (LC-10), UV detector (SPD-10AV) and autoinjector (SIL-10A_{XL}). Mobile phase components (A, B) were as follows: (A) 10 m*M* sodium phosphate buffer (pH 7.5), (B) 10 m*M* sodium phosphate buffer (pH 7.5) containing 40% acetonitrile. Mobile phase flow-rate was 0.5 ml/min, and separation was performed at 35°C. Two connected 2-μm reversed-phase non-porous silica columns, Presto FT-C18 (30×4.6 mm I.D., Imtakt, Kyoto, Japan) were used. A run sequence consisted of a 3-min equilibration with mobile phase A, 1 min of injector activity which concluded with a 10-μl sample injection, a 3-min linear gradient to 5% of mobile phase B followed by a 1-min linear gradient to 100% of mobile phase B, a 4-min hold of those conditions, and a 0.5-min linear gradient back to the initial conditions (mobile phase A). The elute was monitored at 235 nm with a sensitivity of 0.02 a.u.f.s. The chromatographic data were calculated with a Shimadzu CHROMATOPAC C-R7Ae.

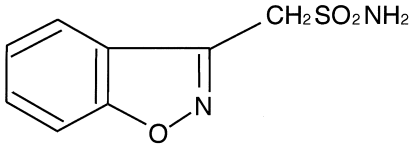

Fig. 1. Structure of zonisamide.

2.3. Standard solutions

A standard stock solution of ZNS was prepared by dissolving 10 mg in 100 ml of methanol. The stock solution of ZNS was stable for 6 months when stored at −20°C. This solution was diluted with methanol to obtain concentrations of 1, 2, 5, 10, 20, 40 and 80 μg/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. ZNS in plasma was stable for at least 3 months at −20°C. The I.S. solution was prepared at 100 μg/ml in 1% acetonitrile in Milli-Q water.

2.4. Sample preparation

The EXtrelut NT1 (5-ml volume) for a column extraction technique followed by HPLC was obtained from Merck (West Point, PA, USA). Plasma (200 μl), 200 μl of 100 m*M* sodium phosphate buffer (pH 5.0) and 100 μl of I.S. solution were applied to the column. ZNS and I.S. were eluted with 6 ml of chloroform. This elute was evaporated to dryness. The residue was dissolved in 200 μl of 10 m*M* phosphate buffer (pH 7.5), containing 1% acetonitrile, and filtered with 0.2-μm membrane filter (Millex-LG, Millipore, Bedford, MA, USA). Then 10 μl was injected onto the HPLC system.

2.5. Precision and accuracy

The precision for the assay of ZNS was examined by performing six replicate analyses in plasma. In order to evaluate the accuracy of the present method for the determination of ZNS in plasma, the drug-free plasma was spiked with 5, 20 and 40 μg/ml of

ZNS. Recovery was determined by comparing the known added amount of the drug with its amount measured by calculating from an adequate standard.

3. Results

Fig. 2 shows representative chromatograms for blank plasma, blank plasma spiked with ZNS, and plasma obtained from patient who was administered ZNS. The retention times of ZNS and I.S. were 2.1 and 3.8 min, respectively. No interfering peaks of endogenous substances were present. The peaks corresponding to ZNS and I.S. were well separated from those of other antiepileptic drugs such as valproic acid, phenobarbital, carbamazepine, phenytoin, clonazepam and nitrazepam (Fig. 2B). No interferences of metabolites, carbamazepine 10, 11-epoxide, 5-(3-hydroxyphenyl)-5-phenylhydantoin, 5-(4-hydroxyphenyl)-5-phenylhydantoin, 7-aminoclonazepam, 7-aminonitrazepam or 7-acetamidonitrazepam were found in this method. These were eluted in the same region as carbamazepine 10,11-epoxide (Fig. 2B). We further studied the possible interferences by supplementing hemolyzed serum (patients receiving no ZNS) with known concentrations of ZNS (20 μg/ml) and the observed concentrations were 21.8±1.5 μg/ml.

A linear correlation was shown between the peak-area ratio of ZNS to I.S. and ZNS concentration in plasma over the range 1–80 μg/ml. The regression equation was $y=0.028\,x+0.011$ ($r^2=0.996$), where y is the peak-area ratio of the drug to the I.S., x is the concentration (μg/ml) of the drug in plasma and r is the correlation coefficient. The lower limits of determination, calculated with $10\times S/N$ ratio, were 0.7 μg/ml for ZNS. This sensitivity of our method extracted from 200 μl of plasma was improved if plasma sample volume was greater than this (limit of detection: 0.1 μg/ml, defined as $3\times S/N$ for 400 μl of plasma).

Table 1
Precision of the assay for ZNS in plasma

ZNS concentration (μg/ml)	Intra-day		Inter-day	
	Recovery[a] (%)	C.V. (%)	Recovery[a] (%)	C.V. (%)
5	102.7±2.8	2.7	106.3±9.5	9.0
20	97.8±6.4	6.6	99.9±3.1	3.1
40	98.2±6.7	6.8	95.4±6.5	6.9

[a] Each value represents the mean±SD of six determinations.

The accuracy test for the determination of ZNS in plasma was examined by adding known amount of ZNS to blank plasma at 5, 20 and 40 μg/ml. The results are summarized in Table 1.

The recoveries for ZNS were 95.4–106.3%. The precision of the determination of ZNS was examined

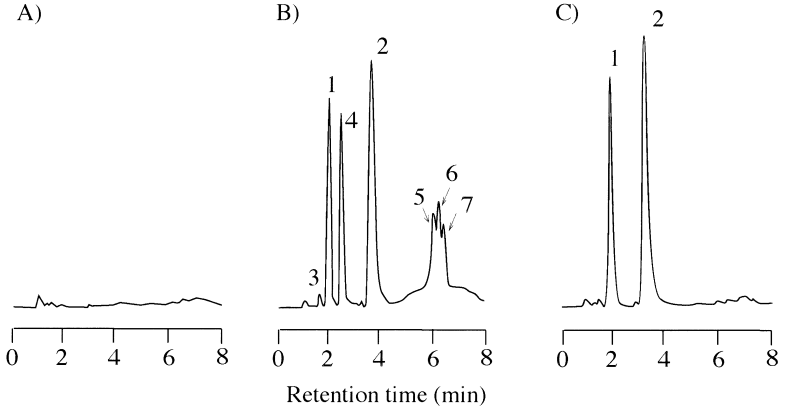

Fig. 2. Chromatograms of (A) a plasma blank, (B) a plasma blank spiked with ZNS (20 μg/ml), methyl *p*-hydroxybenzoate (100 μg/ml), valproic acid (57 μg/ml), phenobarbital (22 μg/ml), carbamazepine 10,11-epoxide (50 μg/ml), carbamazepine (7.1 μg/ml), phenytoin (15 μg/ml), clonazepam (47 ng/ml) and nitrazepam (51 ng/ml), and (C) plasma of a patient receiving ZNS (20.6 μg). Peaks: 1, ZNS; 2, methyl *p*-hydroxybenzoate; 3, valproic acid; 4, phenobarbital; 5, carbamazepine 10,11-epoxide; 6, carbamazepine and phenytoin; and 7, clonazepam and nitrazepam.

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 5, 20 and 40 μg/ml ZNS. Coefficients of variations (C.V.) ranged from 2.7 to 6.8%. For the inter-day precision, 200 μl of quality control plasma, analyzed in six consecutive runs, gave C.V. values in the range of 3.1–9.0%. A summary of these data is given in Table 1.

In Fig. 3, we observed a very good correlation in plasma ZNS concentrations in 25 patients receiving ZNS 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 (the conventional HPLC method: the plasma samples were treated according to the above determination procedure. A 5-μm reversed-phase porous silica column packed with Cosmosil C_{18} (250× 4.6 mm I.D., Nacalai tesque, Tokyo, Japan) was used. The mobile phase was acetonitrile–10 m*M* phosphate buffer (pH 3.0) (35:65, v/v). The flow-rate was 1.0 ml/min, and separation was performed at 40°C. The retention times of ZNS and methyl anthranylate (I.S.) were 5.7 and 15.5 min, respectively). The regression equation was $y=0.973\ x+0.621$ ($r^2=0.998$), where y is the concentration (μg/ml) obtained by the new HPLC method, x is the concentration (μg/ml) obtained by the conventional HPLC method and r is the correlation coefficient.

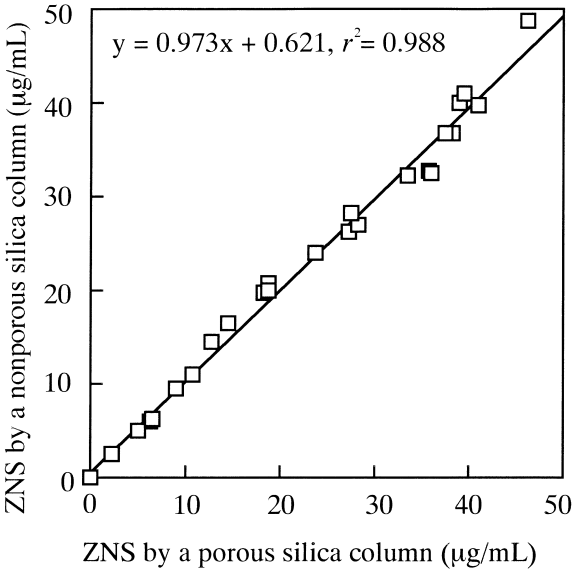

Fig. 3. Comparison of ZNS concentrations obtained in 25 patients by the conventional HPLC method with a porous silica column and the new HPLC method with a non-porous silica column.

4. Discussion

In our evaluation of a new HPLC method for the determination of ZNS concentration in human plasma, the reversed-phase non-porous silica column provided good performance. No appreciable interferences of endogenous substances were detected and detection sensitivity was sufficient to determine ZNS. The replicate analyses indicated low C.V. Both accuracy and precision in the determination of ZNS were satisfactory. In the accuracy test, the measured amounts of ZNS indicated more than 95.4% of the known amount spiked to human plasma. The limit of the determination of ZNS by the present methods was 0.7 μg/ml. The sensitivity of the present method compares favorably with those obtained by other methods [9–13]. It is below the therapeutic range (17–50 μg/ml).

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 [8]. The previous HPLC methods for ZNS needed large amounts of organic modifier as follows: 37% organic modifier, 1.4 ml/min [9], 33% organic modifier, 1.0 ml/min [10], 84% organic modifier, 1.5 ml/min [11], 21% organic modifier, 1.0 ml/min [12], and 9–27% organic modifier, 1.2 ml/min [13]. In our conventional method, ZNS was eluted on the porous silica column using 35% isocratic organic modifier. In contrast, in the new HPLC method by the non-porous silica column, ZNS was eluted with 0.5 ml/min flow-rate, using only 0–2% (0–3 min) organic modifier.

Furthermore, in the new HPLC method by the non-porous silica column, ZNS was eluted up to 2.1 min. In other reports [9–13] and our conventional HPLC method, ZNS was eluted between 3.9 and 7.6 min. ZNS was analyzed up to 1.9–3.1 times faster compared to the porous columns.

The above points demonstrate that the present

method has a significant advantage with respect to reduction of analysis time and saving solvent used without compromising quality. The present method may be useful for routine monitoring or pharmacokinetic studies of ZNS concentrations in patients with epilepsy. We realized good performance from the non-porous silica column and found it to be a valuable addition to the various commercial column options available for method development.

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