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Journal of Chromatography B, 656 (1994) 456–459

JOURNAL OF CHROMATOGRAPHY B: BIOMEDICAL APPLICATIONS

Short Communication

Simple and sensitive assay of zonisamide in human serum by high-performance liquid chromatography using a solid-phase extraction technique

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(First received August 24th, 1993; revised manuscript received February 4th, 1994)

Abstract

A rapid and sensitive method for the assay of zonisamide in serum was developed using a solid-phase extraction technique followed by high-performance liquid chromatography. A 20-μl volume of human serum was first purified with a Bond-Elut cartridge column. Then, the methanol eluate was injected onto a reversed-phase HPLC column with a UV detector. The mobile phase was acetonitrile–methanol–distilled water (17:20:63, v/v) and the detection wavelength was 246 nm. The detection limit was 0.1 μg/ml in serum. The coefficients of variation were 4.2–5.6% and 5.1–9.1% for the within-day and between-day assays, respectively. This method can be used for clinical pharmacokinetic studies of zonisamide in serum even in infant patients with epilepsy.

1. Introduction

Zonisamide (ZNS) is used in the clinical treatment of epileptic disorders as an anticonvulsant drug [1–3]. The therapeutic range of the serum concentration of this drug during dosing is rather narrow; high doses often cause side effects such as ataxia, nystagumus, diplopia, drowsiness, loss of appetite, *etc.* [2–4]. Clinically significant effects are observed with plasma levels higher than 16.5 μg/ml, and some toxic symptoms may occur when the plasma levels exceed 40 μg/ml [2]. Thus, determination of the 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 during chronic drug administration.

The commonly used methods for monitoring the serum concentration of ZNS are gas chromatography (GC) [1,5] and high-performance liquid chromatography (HPLC) [6–8]. HPLC is simpler, more rapid and sensitive than GC. However, the pretreatment of serum or plasma samples in the HPLC method is also rather laborious. Recently, an enzyme-immunoassay method (EIA) for ZNS has been developed [9]. However, it is not easy to obtain an antiserum highly specific for the drug and the enzyme-labeled drug in this method. We describe a more simple and rapid routine method for the monitoring of ZNS in a small sample volume, using a solid-phase extraction technique followed by HPLC.

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SSDI 0378-4347(94)00132-O

2. Experimental

2.1. Chromatographic system

The HPLC system (Waters Assoc., Milford, MA, USA) consisted of a pump (Model 510), UV detector (Model 481 Lambda-Max), autoinjector (Model 712-WISP), and chromatogram data-calculator (Model 710 Data-Module). A stainless steel column (300 × 3.9 mm I.D.) packed with ODS μBondapak C_{18} (10-μm particle size) was used at room temperature. The mobile phase was acetonitrile–methanol–distilled water (17:20:63, v/v) degassed by ultrasonic vibration. The flow-rate was 1.4 ml/min. The detector was set at 246 nm and the sensitivity was 0.02 AUFS.

2.2. Reagents and standard solution

ZNS (3-sulfamoylmethyl-1,2-benzisoxazole) and the internal standard (I.S.) N,N-dimethyl-ZNS were donated by Dainippon Seiyaku (Osaka, Japan). Acetonitrile and methanol, reagent-grade for HPLC, were obtained from Katayama Chemical (Osaka, Japan).

A standard stock solution of ZNS was prepared by dissolving 10 mg in 100 ml of methanol. This solution was diluted with methanol to obtain concentrations of 0.1, 0.3, 1, 3, 10, 30, 60 and 80 μg/ml. A 1-ml volume of each solution was pipeted into a 10-ml test tube, and the methanol was evaporated at 40°C under a stream of nitrogen gas. The residue of each standard was then dissolved in 1 ml of drug-free serum, frozen and stored at −20°C until the assay. The I.S. solution was prepared at 100 μg/ml in 50% methanol.

2.3. Extraction apparatus

A Bond-Elut cartridge column (1 ml volume, Cat. No. 607101) and a Vac-Elut system were obtained from Analytichem International and Varian SPP. (Harbor City, CA, USA).

2.4. Procedure

The Bond-Elut cartridge column, set on the Vac-Elut chamber connected to a vacuum pump, was successively washed twice with 1 ml methanol and twice with 1 ml distilled water. Then, 20 μl of serum, 20 μl of I.S. solution, and 0.8 ml of distilled water were applied to the column. After washing with 1 ml of distilled water, ZNS and I.S. were eluted with 250 μl of methanol. A 40-μl volume of this eluate was injected onto the HPLC system. The column was washed with methanol and distilled water, and repeatedly used for the next sample without further washing.

3. Results and discussion

As shown in Fig. 1, the retention times of ZNS and I.S. were 3.9 and 8.4 min, respectively. No interfering endogenous substances were present. The peaks corresponding to ZNS and I.S. were well separated from those of other antiepileptic drugs such as phenytoin, phenobarbital, carbamazepine and their metabolites occurring in serum samples of patients treated with these antiepileptic drugs for a long period. Valproate was not detected at the 246 nm wavelength used in the present experiment.

An excellent linear correlation was shown between the peak-area ratios of ZNS to I.S. and ZNS concentrations in serum over the range 0.1–80 μg/ml. The regression equation was $y = 29.95x - 0.09$ and the linear regression coefficient (r) was 0.9998. The recovery, calculated by adding ZNS at concentrations of 10, 20 and 40 μg/ml to serum, averaged 102.4% (97.4–106.0%) in ZNS-containing serum and 99.1% (96.0–101.8%) in drug-free serum.

To test the precision of the assay of ZNS in serum, within-day and between-day assays were performed. The coefficients of variation (C.V.s) in within-day assays of serum samples I and II were 4.2% and 5.6% ($n = 10$), respectively (Table 1). The C.V.s of between-day assays of three serum samples including low, medium and

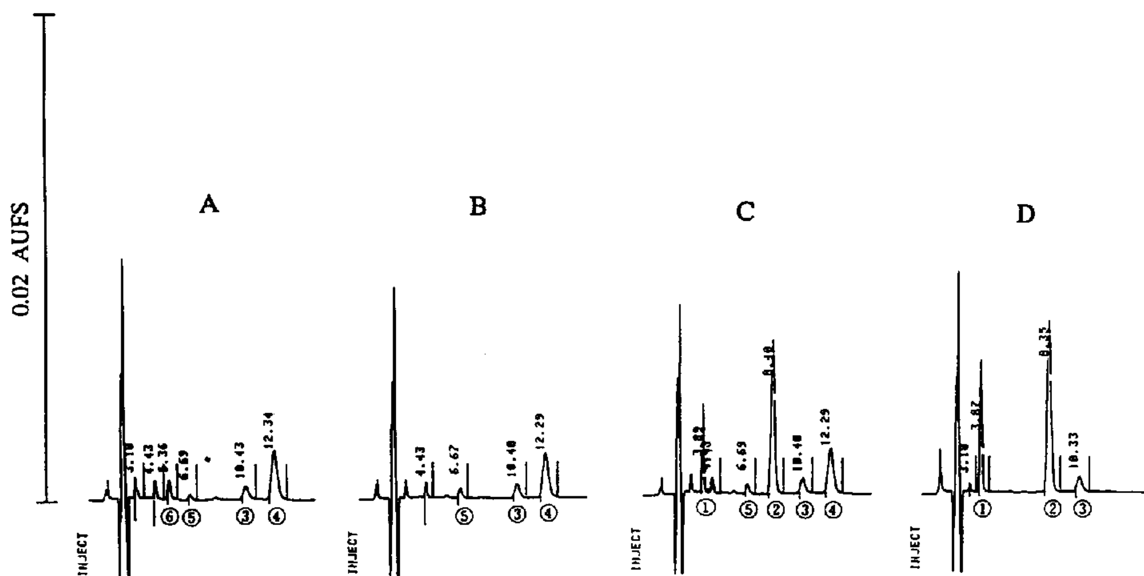

Fig. 1. Separation of zonisamide (ZNS) and internal standard (I.S., N,N-dimethyl-ZNS) from other antiepileptic drugs in serum. Each sample was obtained, 2 h after final drug administration, from patients who showed steady-state serum concentrations of antiepileptic drugs over a long period. (A) Sample containing phenytoin (12.8 μg/ml), phenobarbital (23.5 μg/ml), carbamazepine (7.6 μg/ml), and carbamazepine-epoxide (1.3 μg/ml). (B) Sample containing phenytoin (13.2 μg/ml), carbamazepine (7.4 μg/ml), and carbamazepine-epoxide (1.8 μg/ml). (C) Sample B mixed with ZNS (20 μg/ml) and I.S. (100 μg/ml). (D) Sample containing phenytoin (11.5 μg/ml), valproate (62.6 μg/ml), ZNS (23.6 μg/ml), and I.S. (100 μg/ml). Peaks: 1 = ZNS, 2 = I.S., 3 = phenytoin, 4 = carbamazepine, 5 = carbamazepine-epoxide, 6 = phenobarbital.

high amounts of ZNS obtained from patients with epilepsy were 9.1, 9.0 and 5.1% ($n = 10$), respectively.

Previously reported methods for the assay of serum ZNS include several pretreatment steps prior to injection onto the HPLC system, such as extraction with organic solvent and adjustment of serum pH and evaporation [6,7]. In the present experiment, solid-phase extraction with a Bond-Elut cartridge column containing octadecyl-bonded silica was used as the pretreatment step of the HPLC method for the de-

Table 1
Precision of the assay of ZNS in serum samples

Within-day			Between-day		
Sample	Concentration (mean ± S.D.) (μg/ml)	C.V. (%)	Sample	Concentration (mean ± S.D.) (μg/ml)	C.V. (%)
I	9.6 ± 0.4	4.2	III	5.5 ± 0.5	9.1
II	19.7 ± 1.1	5.6	IV	15.5 ± 1.4	9.0
			V	31.3 ± 1.6	5.1

In the within-day assay, two serum samples were continuously analyzed 10 times, respectively. In the between-day assay, three serum samples from patients administered with different doses of ZNS were divided into 10 parts, respectively, and stored at −20°C until the assay. C.V. = coefficient of variation.

termination of ZNS serum concentrations. This pretreatment procedure is simple and fast, so ten serum samples can be treated within 15 min. In addition, since the cartridge column can be used repeatedly, washing with methanol and distilled water after a run is sufficient to prepare the column for the next sample. The C.V.s were less than 5.6 and 9.1% in within-day and a between-day assays, respectively.

In the monitoring of therapeutic drugs, the blood volume collected from a patient is one of the most important factors. In particular, it is not easy to obtain large volume blood sample from small infants. In this study, the ZNS concentrations could be determined in only 20 μl of serum, which can be obtained from approximately 50 μl of whole blood using a hematocrit capillary tube.

In conclusion, the present method may be useful for routine monitoring or pharmacokinetic studies of serum ZNS concentrations even in small infant patients with epilepsy.

4. References

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