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Short communication

Simultaneous determination of zonisamide, carbamazepine and carbamazepine-10,11-epoxide in infant serum by high-performance liquid chromatography

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Abstract

This study developed a simple method for the simultaneous determination of zonisamide (ZNS), carbamazepine (CBZ) and its active metabolite, carbamazepine-10,11-epoxide (CBZE) in infant serum using reversed-phase high-performance liquid chromatograph (HPLC). The method involves a single-step protein precipitation procedure that uses no solid-phase or liquid–liquid extraction. The HPLC separation was carried out on a Cadenza CD-C18 column (3 μm, 4.6 mm × 150 mm) with potassium phosphate buffer (pH 4.6; 25 mM)–methanol–acetonitrile (65:20:15 (v/v/v)) as a mobile phase at a 1.0 ml/min flow rate: ZNS was detectable using a UV detector at 235 nm, and both CBZ and CBZE were at 215 nm. The quantification limits were established in accordance with each therapeutic range at 2.5 μg/ml for ZNS, 0.5 μg/ml for CBZ, and 0.25 μg/ml for CBZE. The respective coefficients of variation were 1.3–6.0% and 2.2–7.7% for the intra- and inter-assay.
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1. Introduction

Zonisamide (ZNS; 3-sulphamoylmethyl-1,2-benzisoxazol) is used as an antiepileptic drug (AED) in the clinical treatment of epileptic disorders [1,2]. The therapeutic range of the serum concentration of ZNS is usually attained with plasma concentrations of 17–50 μg/ml. Some toxic symptoms might occur with plasma concentrations greater than 40 μg/ml [1]. Clinically, ZNS is often used in combination with other AEDs, especially carbamazepine (CBZ) [3].

In therapeutic drug monitoring for CBZ, we recommend determination of carbamazepine-10,11-epoxide (CBZE), as well as its parent drug CBZ because CBZE is found to have an antiepileptic activity. It is also partially responsible for the side effects of CBZ therapy [4]. Optimal therapeutic levels of CBZ in serum were 4–12 mg/l; those levels for CBZE, which was intoxicated with CBZ, were 0.8–3.2 mg/l [5].

Many high-performance liquid chromatographic (HPLC) methods have been published for determination of ZNS alone [6–8], or for CBZ, which contains its important metabolite, CBZE [9–12], in biological fluid. However, in spite of their general co-administration to epileptic patients, few methods have been proposed for simultaneous determination of ZNS and CBZ. This paper presents a description of a simple and rapid routine method for simultaneous monitoring of ZNS, CBZ and CBZE in sera of infant patients with epilepsy using reversed-phase HPLC techniques.

2. Experimental procedures

2.1. Chemicals

Dai Nippon Pharmaceutical Co. Ltd. (Osaka, Japan) kindly supplied ZNS. CBZ was purchased from Kanto Chemical Co.

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Inc. (Tokyo, Japan). CBZE, sodium valproic acid, sodium 5,5-diphenylhydantoin, and chlorzoxazone (as an internal standard) of analytical grade were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Sodium phenobarbital was from Wako Pure Chemical Industries Ltd. (Osaka, Japan). Acetonitrile, methanol and distilled water were of HPLC grade. Other chemicals were of analytical-regent grade. Drug-free serum was commercially available from Bio-Rad Laboratories Inc. (Hercules, CA, USA).

2.2. Standard solutions

Stock standard solutions of ZNS and CBZ were prepared by dissolving 100 mg in 100 ml of methanol; CBZE was also dissolved in methanol, thereby preparing a stock solution of 100 μg/ml. These solutions were mixed and diluted with methanol as working standard solutions at six respective concentrations of 2.5, 5, 10, 20, 40, and 80 μg/ml of ZNS, 0.5, 1, 2, 4, 8, and 16 μg/ml of CBZ, and 0.25, 0.5, 1, 2, 4, and 8 μg/ml of CBZE.

A 100-μl aliquot of each of the six different concentrations of working standard solutions was pipetted and transferred into a 1.5 ml centrifuge tube; the methanol was evaporated to dryness using a nitrogen stream. The residue of each standard was then dissolved in a 100-μl aliquot of drug-free serum, frozen and stored at −20 °C until analyses. These standard solutions for construction of calibration curves in sera were stable at −20 °C for at least 2 months. The chlorzoxazone solution, which was used as an internal standard and a deproteinisation solvent, was prepared at 20 μg/ml in acetonitrile and stored at −20 °C until analyses. The internal standard solution was also stable at −20 °C for at least 2 months.

2.3. Apparatus and chromatographic conditions

Analyses of standards and sample extracts were conducted using a liquid chromatography system (1090 series II; Agilent Technologies Inc., Waldbronn, Germany) including a pump, a photodiode array detector, a column thermostat, and an automatic sample injector. The analytical column (3 μm particle size, 150 mm × 4.6 mm i.d., Cadenza CD-C18; Imtakt Corp., Kyoto, Japan) was heated at 50 °C during the analyses. The mobile phase was potassium phosphate buffer (pH 4.6; 25 mM)–methanol–acetonitrile (65:20:15 (v/v/v)) at a flow rate of 1.0 ml/min under isocratic conditions. The injection volume was 20 μl; the UV detector wavelengths were 215 nm for determination of CBZ and CBZE, and 235 nm for ZNS. Fig. 1 shows typical chromatograms obtained, respectively, from the stock standard solution and calibration standard in serum with detection at 215 and 235 nm.

2.4. Sample preparation

A 100-μl aliquot of a serum sample in a 1.5 ml centrifuge tube was added to 100 μl of acetonitrile containing chlorzoxazone (20 μg/ml) as the internal standard and deproteinisation solvent. The solution was vortex-mixed for a few seconds and kept at room temperature for 5 min. The mixture was then centrifuged for 10 min at 13,500 rpm. Subsequently, 20-μl of the clear supernatant was injected into the HPLC system. Six serum standard solutions for construction of the calibration curves were added to 100 μl of acetonitrile containing I.S., following the preparation method described above.

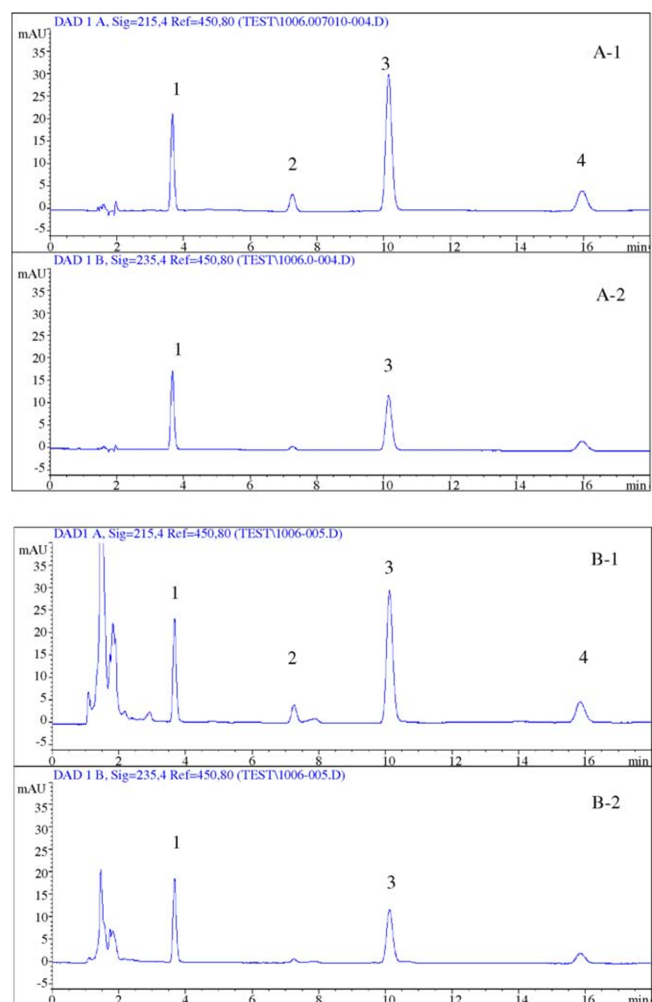

Fig. 1. Typical chromatograms of a working (A) and a serum standard solution (B) containing 10.0, 2.0 and 1.0 μg/ml of ZNS, CBZ and CBZE. (A-1 and B-1, detection at UV215 nm; A-2 and B-2, at 235 nm). Peaks: 1, ZNS; 2, CBZE; 3, chlorzoxazone (I.S., 20 μg/ml); and 4, CBZ.

2.5. Precision and accuracy

The intra-assay precision and accuracy of the proposed method were evaluated by performing six replicate analyses of control samples with four different concentrations. Performing analyses of the same control samples assessed the inter-assay precision and accuracy. The procedures were repeated on eight different days.

3. Results

3.1. Chromatography

Typical chromatograms resulting from analyses of various serum samples are shown in Fig. 2. To investigate possi-

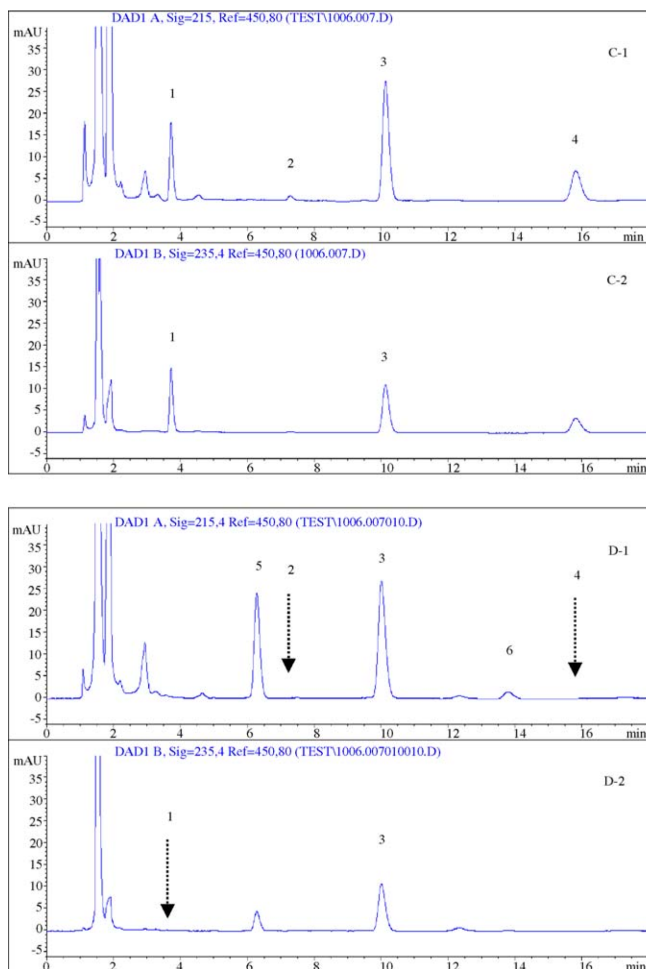

Fig. 2. Typical chromatograms of a serum sample collected from an epileptic patient with ZNS and CBZ co-medicated (C), and a serum from a patient medicated to Phenobarbital (PB), 5,5-diphenylhydantoin (PHT), varproic acid and clobazam without ZNS and CBZ (D) (C-1 and D-1, detection at UV 215 nm; C-2 and D-2, at 235 nm). Peaks: 1, ZNS (8.80 μg/ml); 2, CBZE (0.27 μg/ml); 3, I.S.; 4, CBZ (3.34 μg/ml); 5, PB; and 6, PHT.

ble chromatographic interferences by drugs co-administrated to epileptic patients, 30 samples of patients under drug therapy without ZNS and CBZ were analyzed. No chromatographic interference from endogenous substances or other AEDs such as 5,5-diphenylhydantoin, valproic acid, phenobarbital, clobazam, primidone, diazepam, clonazepam, nitrazepam and their metabolites, were observed.

3.2. Precision and accuracy

The intra- and inter-assay precision and accuracy of the proposed method were evaluated. The results are shown in Table 1. Precision is expressed as coefficients of variation (CV) of 1.31–6.04% for the intra-assay, and 2.19–7.67% for the inter-assay. Accuracy is defined as (found value/added value) × 100%, which was approximately 100% for each drug throughout examinations for the four different concentrations.

3.3. Linearity

Linearity was tested through analyses of serum calibration standards containing known amounts of six different concentrations of each drug. Calibration curves were linear in the concentration ranges of 2.5–80 μg/ml for ZNS, 0.5–16 μg/ml for CBZ, and 0.25–8 μg/ml for CBZE. The correlation coefficient (r) was higher than 0.998 for all calibration curves.

3.4. Limit of quantification

Concentration ranges of calibration curves in sera were designed in accordance with each therapeutic range. The quantification limits were set as the lowest drug concentrations, below 1/3 of the levels of therapeutic ranges, such as 2.5 μg/ml for ZNS, 0.5 μg/ml for CBZ, and 0.25 μg/ml for CBZE, at which the signal-to-noise ratios of the HPLC peaks were at least 5:1 and good intra- and inter-assay CV values (<7.67%) and recoveries (95.1–98.5%) were achieved for calibration standards (Table 1).

Table 1
Intra- and inter-assay precision and accuracy results for serum samples with ZNS, CBZ, and CBZE

Drugs	Added (μg/ml)	Intra-assay ($n=6$)			Inter-assay ($n=8$)		
		Found (mean ± S.D.) (μg/ml)	Precision CV (%)	Accuracy (%)	Found (mean ± S.D.) (μg/ml)	Precision CV (%)	Accuracy (%)
ZNS	2.5	2.40 ± 0.09	4.11	96.1	2.38 ± 0.09	3.93	95.1
	10.0	10.08 ± 0.16	1.58	100.8	10.33 ± 0.24	2.34	103.3
	20.0	20.03 ± 0.26	1.31	100.2	20.58 ± 0.60	2.92	102.9
	40.0	40.35 ± 0.65	1.61	100.9	40.85 ± 0.89	2.19	102.1
CBZ	0.5	0.48 ± 0.02	4.79	96.3	0.49 ± 0.04	7.67	97.9
	2.0	2.10 ± 0.07	3.20	105.1	2.03 ± 0.09	4.49	101.3
	4.0	3.95 ± 0.09	2.28	98.7	4.07 ± 0.15	3.76	101.8
	8.0	8.00 ± 0.13	1.56	100.0	8.06 ± 0.21	2.55	100.8
CBZE	0.25	0.25 ± 0.02	6.04	98.4	0.25 ± 0.02	7.64	98.5
	1.0	1.02 ± 0.03	3.16	102.0	1.02 ± 0.08	7.45	101.9
	2.0	2.01 ± 0.04	2.05	100.4	2.04 ± 0.10	4.75	102.0
	4.0	4.09 ± 0.06	1.47	102.1	4.06 ± 0.10	2.53	101.5

Table 2
Absolute recoveries of ZNS, CBZ, and CBZE from human serum

Drugs ($n = 6$)	Concentration (μg/ml)	Recovery (mean ± S.D.) (%)	CV (%)
ZNS	10.0	102.9 ± 2.7	2.62
	20.0	103.8 ± 2.6	2.53
	40.0	102.8 ± 1.7	1.65
CBZ	2.0	101.7 ± 4.9	4.84
	4.0	102.4 ± 2.8	2.76
	8.0	100.7 ± 3.0	3.01
CBZE	1.0	100.0 ± 5.7	5.73
	2.0	102.9 ± 5.3	5.19
	4.0	101.5 ± 3.0	2.99

3.5. Absolute recovery

Absolute recoveries for ZNS, CBZ, and CBZE were calculated at three different concentrations by comparing the peak area of the serum standard solution with the mean peak area obtained from direct injection of the corresponding standard solutions added to the I.S. solution. Table 2 lists the sufficient results of recovery studies.

3.6. Routine analysis

Table 3 shows the results of ZNS, CBZ, and CBZE concentrations from out-patients during routine therapeutic drug monitoring. There had been no change in dose (ZNS and CBZ) for at least 1 month before serum drug analyses.

4. Discussion

Clinically, ZNS is administered alone in only a few cases; it is used mostly in combination with other AEDs, especially CBZ. In spite of their widespread co-administration to epileptic patients, separate methods have been adopted [13]. Few methods have been published for the simultaneous determination of ZNS and CBZ relatives.

Most published methods for determination of ZNS alone or CBZ relatives in biological fluids use solid-phase [6,8,14] or liquid–liquid extraction [7,9,12,13,16]. They often use evaporation of the organic extract under an air [7] or a nitrogen stream [6,12–16] in sample preparation. In fact, those preparation procedures for samples are time-consuming, rendering them unavailable for routine drug monitoring. In contrast, sample preparation procedures involving a direct deproteinisation with acetonitrile are simple and rapid. Furthermore, it avoids loss and degradation of the target drugs. Using this pretreatment procedure, 10 samples can be treated within 30 min: it is readily available for routine drug monitoring.

Investigations were undertaken for optimal chromatographic condition, choice of UV wavelengths, various mobile phase compositions containing pH value adjustment, and analytical columns. First, a single UV monitoring at 215 nm was adopted for analyses; however, some impurity peaks from patient samples were observed near the ZNS peak. Therefore, two wavelength-monitoring methods at 215 and 235 nm were chosen considering the sensitivities of ZNS and CBZ relatives.

Chromatographic resolution of phenobarbital from ZNS was sensitive to changes in pH value of phosphate buffer used as a mobile phase. At pH 7.0, inadequate separation between peaks was obtained. Adjustment to pH 4.6 enabled a good separation between the drugs, as shown in Fig. 2(C-1, D-1). Results showed that the retention time of phenobarbital increased as the pH value of the phosphate buffer decreased. On the other hand, the choice of the Cadenza CD-C18 analytical column enabled the peaks to be sharpened and heightened in comparison with other commercially available HPLC columns using amide C_{16}, C_8 or C_{18} materials as the stationary phase. Furthermore, adequate separation of the peak of ZNS from that which was mostly observed from the patient serum with CBZ co-medicated was attained easily and completely using this column. The specific peak was inferred to be another CBZE metabolite, CBZ trans-diol. Under these conditions, no chromatographic interference

Table 3
ZNS, CBZ, and CBZE concentrations (μg/ml) from out-patients during routine therapeutic drug monitoring

Pt no.	ZNS	CBZ	CBZE	Other AEDs
1	21.03 ± 0.15	8.81 ± 0.06	1.75 ± 0.07	Clobazam
2	14.25 ± 0.12	4.33 ± 0.07	0.94 ± 0.05	Valproic acid
3	8.41 ± 0.10	5.30 ± 0.07	0.75 ± 0.04	
4	21.95 ± 0.14	6.94 ± 0.07	1.00 ± 0.04	Clobazam
5	14.36 ± 0.11	5.08 ± 0.08	0.98 ± 0.06	Clonazepam
6	12.35 ± 0.09	5.35 ± 0.08	0.85 ± 0.03	
7	10.68 ± 0.08	5.21 ± 0.04	1.44 ± 0.05	Phenobarbital, phenytoin
8	8.01 ± 0.04	4.09 ± 0.05	0.50 ± 0.05	Phenobarbital, clonazepam

Data are given as mean ± S.D. ($n = 3$).

from endogenous substances or other AEDs and their metabolites was observed throughout the analyses.

For routine monitoring of therapeutic drugs, the blood volume collected from a patient is an important consideration. Particularly from small infants, it is not easy to obtain a large volume of blood. In this study, ZNS and CBZ relatives were determined from 100 μl of serum, which is half of the sample volume proposed in a previous study [6]. Moreover, the replicate analyses yielded a low CV. Both accuracy and precision in the determination of ZNS and CBZ relatives were also satisfactory.

Therefore, this proposed method is useful for routine drug monitoring or pharmacokinetic studies of ZNS and CBZ relatives in infant epileptic patients.

5. Conclusion

A simple method was developed for simultaneous determination of ZNS, CBZ, and its active metabolite, CBZE, in serum. The method uses reversed-phase liquid chromatography with a single-step protein precipitation procedure and a smaller sample volume collected from patient. Results obtained by this study demonstrate its usefulness for determination of ZNS and CBZ relatives. It is particularly feasible for routine monitoring or pharmacokinetic studies of ZNS, CBZ, and CBZE concentrations in infant patients with epilepsy.

References

[1] J.C. Sackellares, P.D. Donofrio, J.G. Wagner, B. Abou-Khalil, S. Berent, K. aasved-Hoyt, Epilepsia 26 (1985) 206–211.
[2] A.J. Wilensky, P.N. Friel, L.M. Ojemann, C.B. Dodrill, K.B. McCormick, R.H. Levy, Epilepsia 26 (1985) 212–220.
[3] T. Minami, I. Ieiri, K. Ohtsubo, Y. Hirakawa, K. Ueda, S. Higuchi, T. Aoyama, Epilepsia 35 (1994) 1023–1025.
[4] B. Rambeck, A. Salke-Treumann, T. May, H.E. Boenigk, Eur. Neurol. 30 (1990) 79–83.
[5] R. Kanarkowski, J. Rybakowski, Psychiatr. Pol. 5–6 (1989) 379–386.
[6] M. Nakamura, K. Hirade, T. Sugiyama, Y. Katagiri, J. Chromatogr. B 755 (2001) 337–341.
[7] D.J. Berry, J. Chromatogr. B 534 (1990) 173–181.
[8] K. Furuno, R. Oishi, Y. Gomita, K. Eto, J. Chromatogr. B 656 (1994) 456–459.
[9] J. Martens, P. Banditt, J. Chromatogr. B 620 (1993) 169–173.
[10] D. Chollet, E. Castella, P. Combe, V. Arnera, J. Chromatogr. B 683 (1996) 237–243.
[11] J.M. Potter, A. Donnelly, Ther. Drug Monit. 20 (1998) 652–657.
[12] L.A. Romanyshyn, J.K. Wichmann, N. Kucharczyk, R.C. Shumaker, D. Ward, R.D. Sofia, Ther. Drug Monit. 16 (1994) 90–99.
[13] I. R-Majlessi, R.H. Levy, D. Bergen, W. Garnett, W. Rosenfeld, G. Mather, J. Shaj, J.S. Grundy, Epilepsy Res. 62 (2004) 1–11.
[14] G. Minkova, D. Getova, Methods Find Exp. Clin. Pharmacol. 23 (2001) 481–485.
[15] D.A. Svinarov, C.E. Pippenger, Ther. Drug Monit. 17 (1995) 217–220.
[16] J. Bonneton, P. Genton, E. Mesdjian, Biopharm. Drug Dispos. 13 (1992) 411–416.