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Short Communication

Validation of the analysis of carbamazepine and its 10,11-epoxide metabolite by high-performance liquid chromatography from plasma: comparison with gas chromatography and the enzyme-multiplied immunoassay technique

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ABSTRACT

An improved procedure for the determination of carbamazepine (CBZ) and its main active metabolite carbamazepine-10,11-epoxide (CBZ-e) in serum is described. The validation of this procedure shows that the limits of detection are lower than 80 ng/ml for CBZ and 7 ng/ml for CBZ-e. The relative standard deviation does not exceed 4.7% for both compounds in an inter-day precision test. In the intra-day precision test the relative standard deviations are 1.9% for CBZ-e and 1.1% for CBZ. A comparison of the described procedure with gas chromatography and the enzyme-multiplied immunoassay technique shows good agreement, but gas chromatography seems to have a relative poor reliability.

INTRODUCTION

Carbamazepine (CBZ) is an established drug for control of grand mal and psychomotor epilepsy and it is also effective in the treatment of trigeminal neuralgia. Furthermore, it is nowadays frequently used in bipolar depression [1]. It is predominantly eliminated in the liver, where it is metabolized to carbamazepine-10,11-epoxide (CBZ-e) and other derivatives [2]. CBZ-e seems to have antiepileptic properties as well as CBZ itself [3], and it is thought to be partially responsible for the side effects of a CBZ-therapy [4]. Because the metabolic rate depends highly on the presence of enzyme inducers, such as primidone, phenytoin or phenobarbitone [5], which are in common in antiepileptic therapy, it is recommended to determine CBZ-e as well as its parent drug CBZ in therapeutic drug monitoring (TDM). There are several HPLC-procedures which deal with the determination of antiepileptic drugs. Most of them are capable to separate all usual antiepileptic drugs, but they use the gradient elution technique and therefore need expen-

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sive equipment and are quite time consuming [6,7]. A very easy extraction and HPLC-detection procedure for CBZ and CBZ-e was described in a recent paper [8]. In our work we have adapted this procedure for our equipment, optimized it and performed a method-validation, including an inter-method comparison with an enzyme-multiplied immuno assay technique (EMIT)- and GC-procedure.

EXPERIMENTAL

HPLC procedure

Apparatus. The HPLC system used in this work was a Gynkotek Model 300 (Munich, Germany). Samples were injected manually into a 20-μl sample loop. The column was a LKB-Pharmacia (Freiburg, Germany) Spherisorb ODS-2 (250 mm × 4 mm I.D., 5 μm) with a guard-column LKB-Pharmacia Spherisorb ODS-2 (10 mm × 4 mm I.D., 5 μm). The effluent was monitored with a Gynkotek Model SP 6 UV-detector. The data were collected and analyzed by a Gynkotek C-R 6 A integrator.

Chemicals. Carbamazepine and theophylline were purchased from Arzneimittelwerk Dresden (Dresden, Germany), carbamazepine-10,11-epoxide from Ciba-Geigy (Basel, Switzerland), ethyl acetate, chloroform, acetonitrile, hexane, sodium chloride and sodium hydroxide were P.A. grade from Merck (Darmstadt, Germany). The internal standard was 1,3-dimethyl-7-benzylxanthine, which was synthesized from theophylline and bromobenzene in the presence of potassium carbonate with boiling benzene as the solvent. Control (blank) human sera were obtained from healthy volunteers.

Sample preparation. Serum (400 μl) was mixed with 50 μl of standard solution containing 100 μg/ml of 1,3-dimethyl-7-benzylxanthine in water. NaOH (400 μl, 1.5 *M*) solution and 100 mg of NaCl were added. This mixture was extracted with 4 ml of ethyl acetate–chloroform (1:1, v/v) by shaking for 15 min. After centrifugation the supernatant (organic phase) was evaporated and the residue reconstituted with 200 μl of hexane and 200 μl of mobile phase. After mixing, 20 μl of the mobile phase were injected into the HPLC system.

Chromatographic conditions. The analysis was performed with an isocratic flow-rate of 1.1 ml/min of a mixture of acetonitrile–water (3:7, v/v). The column was held at room temperature and the effluent was monitored at a wavelength of 210 nm. The retention times for CBZ-e, the internal standard and CBZ were 5.6 min, 8.2 min and 10.9 min, respectively.

GC and EMIT procedure

The GC-system was a Hewlett-Packard HP.5890A with autosampler, purged packed inlet, HP-17 capillary column (10 m × 0.53 mm I.D.), and flame ionization detector (FID). The data acquisition and integration were made with an HP.3396 series II integrator. Sample preparation was performed according to ref. [9].

The immunoassay was a SYVA carbamazepine assay run on a SYVA Autolab System. Sample handling was performed according to the SYVA instructions.

RESULTS AND DISCUSSION

Chromatography

The use of 1,3-dimethyl-7-benzylxanthine as the internal standard has been choosen carefully. It has the same extraction properties as the analytes and its retention time was exactly between those of CBZ and CBZ-e. It is truly a non-biomolecule, and hence interferences with other substances are unlikely and up till now not observed by us.

A list of possible interfering substances is given in ref. [8]. No interferences are observed there. We have found phenytoin as a potentially interfering substance, because it can be extracted with the procedure described and its retention time is close to the retention time of CBZ. A test showed, that the quantification of CBZ is not disturbed. Typical chromatograms are shown in Fig. 1.

The detection wavelength of 210 nm is the best compromise between the absorption maxima of CBZ and CBZ-e and gives a slightly higher re-

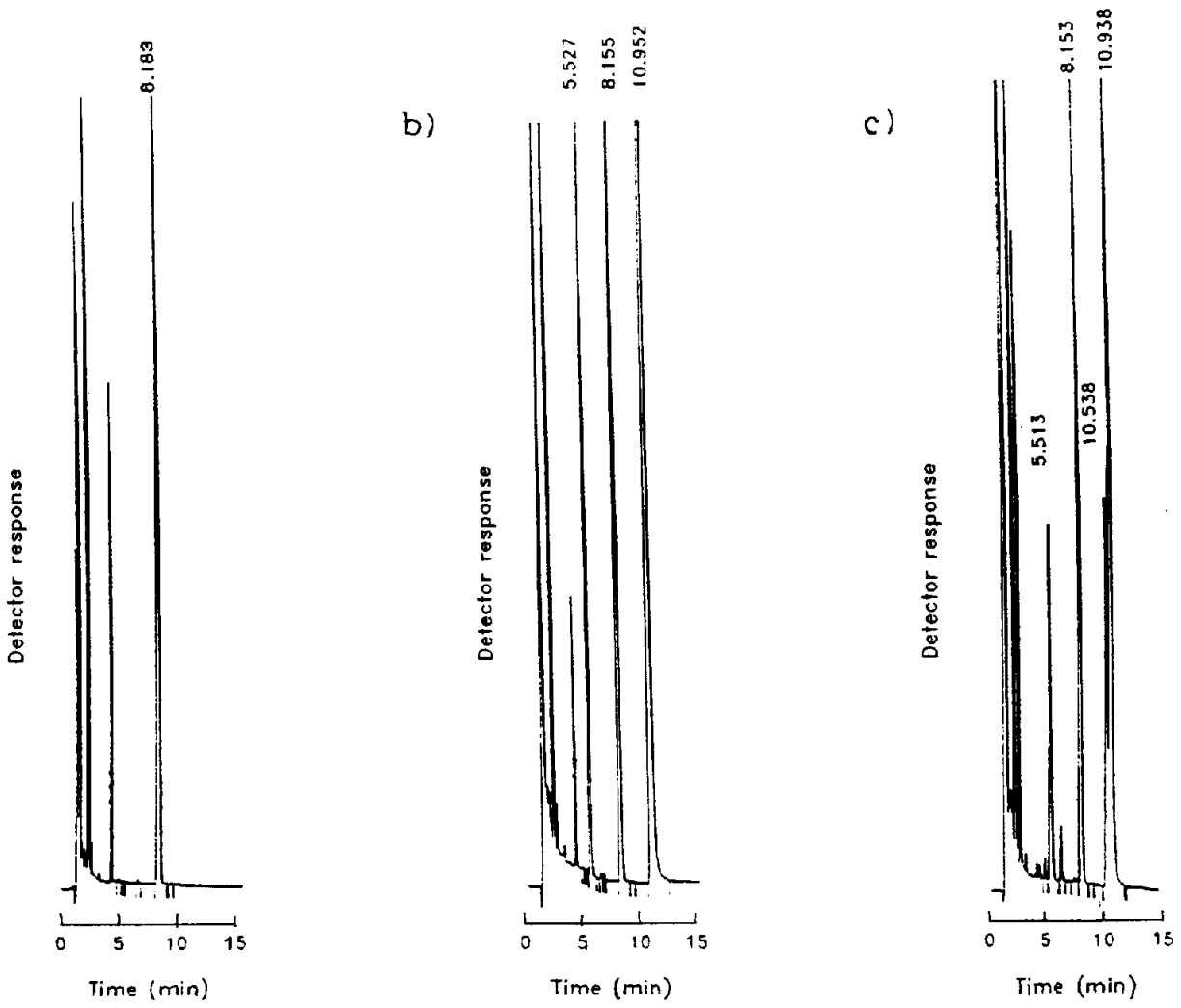

Fig. 1. (a) Blank serum spiked with I.S. (t_R = 8.183). (b) Blank serum spiked with 9 μg/ml CBZ (t_R = 10.952), 1.8 μg/ml CBZ-e (t_R = 5.527) and I.S. (t_R = 8.155). (c) Patient sample containing phenytoin (t_R = 10.538) and CBZ (t_R = 10.938), CBZ-e (t_R = 5.513) and I.S. (t_R = 8.153).

sponse (slope of the calibration curve) for CBZ-e. This is beneficial because usually CBZ-e is found in lower concentrations than CBZ.

Validation

The results for the calibration samples are given in Table I. As can be seen the calibration functions are linear over the tested range and have good regression parameters.

The limits of detection and quantification are derived from multiple measurements in the low concentration range. The values found are extrapolated to the point, where, with a confidence of 95%, the detector signal is higher than the signal from a blank sample. This concentration value is defined as the limit of detection. Multiplication by 3.5 yields the limit of quantification, where the results have an uncertainty of 33%. The resulting limits are very low and suitable for trace studies.

The results of the inter-day and intra-day precision tests are given in Table II. The assay is stable over a period of several days and does not need recalibration.

TABLE I

CALIBRATION DATA AND LIMITS OF DETECTION

Compound	Range (n = 10) (μg/ml)	Intercept	Slope	r	Limit of detection (μg/ml)	Limit of quantification (μg/ml)
CBZ	1.0–10.0	−0.0167	0.9997			
	0.2– 2.0	−0.0036	0.2265	0.9986	0.08	0.27
CBZ-e	0.2– 2.0	0.0042	0.2617	0.9996		
	0.02– 0.2	−0.0009	0.2652	0.9989	0.007	0.025

TABLE II

PRECISION AND ACCURACY DATA FOR CBZ AND CBZ-e

Compound	Concentration (μg/ml)	Intra-day (n = 10)		Inter-day (n = 10)	
		Mean ± S.D. (μg/ml)	R.S.D. (%)	Mean ± S.D. (μg/ml)	R.S.D. (%)
CBZ	1.0	1.12 ± 0.012	1.0	1.05 ± 0.032	3.1
	10.0	10.24 ± 0.115	1.1	10.40 ± 0.279	2.7
CBZ-e	0.2	0.19 ± 0.004	1.9	0.21 ± 0.010	4.7
	2.0	2.15 ± 0.031	1.8	2.16 ± 0.072	3.3

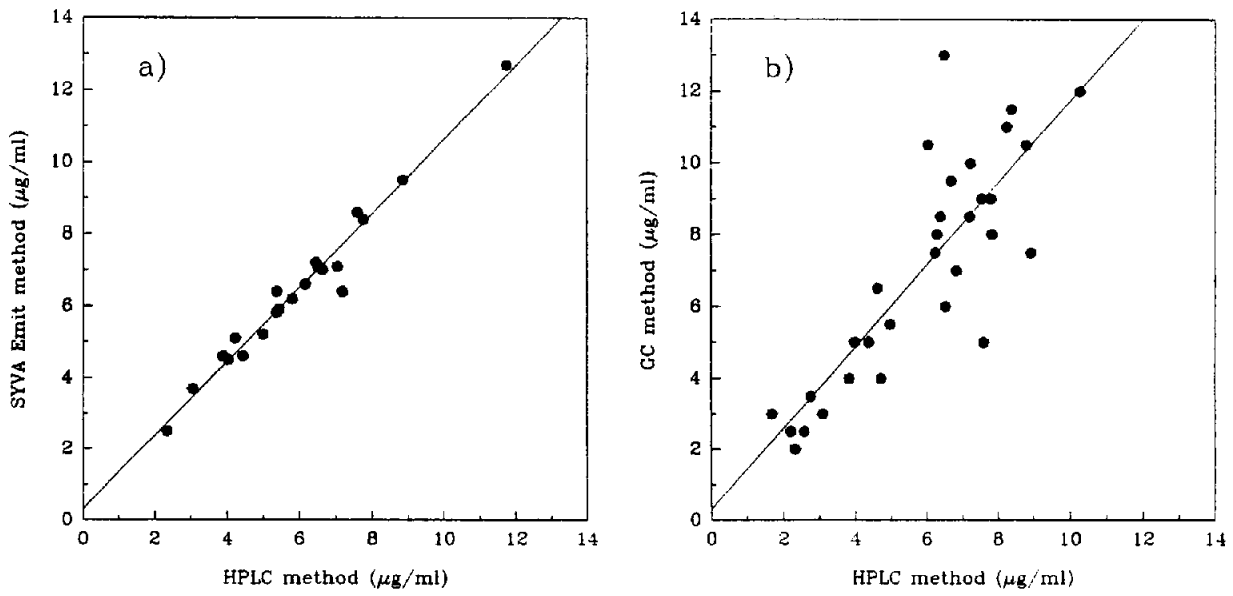

Fig. 2. (a) Correlation between the CBZ levels detected by HPLC and EMIT (n = 21, y = 0.3122 + 1.0312x, r = 0.9835). (b) Correlation between the CBZ levels detected by HPLC and GC (n = 34, y = 0.2972 + 1.1419x, r = 0.8392).

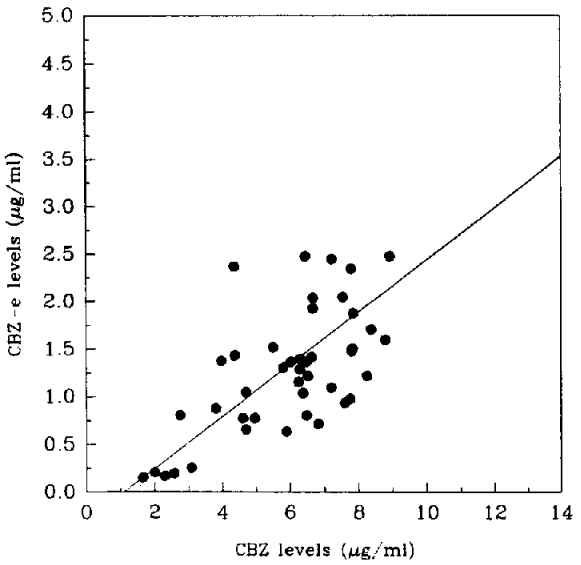

Fig. 3. Relationship between the CBZ and CBZ-e serum levels in epileptic patients ($n = 34$, $y = -0.4691 + 0.2960x$, $r = 0.768$).

Inter-method comparison

Analysis of CBZ with the method described has been compared with the analog determinations by the EMIT and a GC assays. The analysis of CBZ-e unfortunately cannot be compared, because there is no suitable GC assay for CBZ-e and the EMIT assay is not sensitive for this compound. Comparison of HPLC and EMIT (Fig. 2a) shows a very good agreement in the results with a slope of 1.0312, an intercept of 0.3122 and a correlation coefficient of 0.9835. The accuracy of the HPLC assay is thus confirmed. Fig. 2b shows the comparison between HPLC and GC. The slope is 1.1419, the intercept 0.2972 and the correlation coefficient 0.8392. The correspondence is quite poor, because of the complicated GC procedure, which includes a pre-column derivatization step in the injection-line with an unpredictable yield.

Application of the method in TDM

Serum samples of 34 epileptic patients, who were treated with CBZ and partly with other antiepileptic drugs, have been analyzed by this procedure and the CBZ and CBZ-e serum levels were monitored. Fig. 3 shows the proportion of the two compounds. The slope of the regression line is 0.3. This is similar to the value given as a normal value in ref. [2] in the steady state. No severe interference with other compounds has been observed.

CONCLUSION

The procedure described is an easy, accurate, sensitive and cheap assay for CBZ and CBZ-e. Although cross-validation for CBZ-e could not be performed, the other validation parameters are very satisfying and thus the results are thought to be reliable. The assay can be performed in any laboratory even without an expensive EMIT-station (which cannot determine CBZ-e) or a sophisticated HPLC-equipment. With an autosampler it is possible to analyze about 100 samples a day, prepared by one medical assistent.

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