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High-speed liquid chromatographic method for the monitoring of carbamazepine and its active metabolite, carbamazepine-10,11-epoxide, in human plasma

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Abstract

The assays of antiepileptic drugs, which are performed by central laboratories in Phase II and III clinical trials, require both a very fast turn-around time and a suitable specificity. In order to decrease the run time and to keep the powerful specificity of the liquid chromatography (HPLC), the use of a reversed-phase 1.5 μm monosized non-porous silicon dioxide microspheres column instead of regular columns containing spherical porous C_{18} material was studied. The determination of carbamazepine (CBZ) and its active metabolite, carbamazepine-10,11-epoxide (CBZ-E), in human plasma or serum was chosen to demonstrate the utility of these columns. As a prerequisite of this work, no modification of a regular HPLC system was allowed. The samples were prepared in autosampler vials by protein precipitation with acetonitrile, followed by a quick centrifugation. Without any change to a conventional HPLC system, CBZ and CBZ-E are well separated in less than 2.5 min using a Kovalis MS C_{14} column. No interference was observed with endogenous compounds and with nine antiepileptic drugs commonly prescribed as co-medication, and their metabolites. Due to the very low specific surface area of the packing, the required organic modifier volume per chromatographic run was decreased by a factor of 25. The method was validated. The developed method is well suited for the determination of CBZ and CBZ-E in clinical trials. It can be easily adapted to the monitoring of other antiepileptic drugs. No modification of a regular HPLC system was required.

Keywords: Carbamazepine; Carbamazepine-10,11-epoxide

1. Introduction

Carbamazepine (CBZ) is an established antiepileptic drug (AED) that is widely used as monotherapy and as co-medication in the treatment of both partial and generalized tonic-clonic seizures [1]. CBZ is metabolized to its metabolite carbamazepine-10,11-epoxide (CBZ-E). CBZ-E seems to have antiepileptic activity, as does CBZ itself, and it is

thought to be partially responsible for the side effects of CBZ therapy. CBZ-E is further metabolized to the inactive 10,11-dihydro-*trans*-dihydroxycarbamazepine. The metabolic rate depends highly on the presence of enzyme inducers. Under steady-state conditions, plasma concentrations of 13–55 $\mu\text{mol/l}$ and 3–23 $\mu\text{mol/l}$ were reported for CBZ and CBZ-E, respectively [2]. The *trans*-diol metabolite can reach plasma concentrations even higher than those of CBZ-E [3].

Many methods have been proposed for the

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simultaneous determination of CBZ and CBZ-E in biological fluids (for review see Ref. [4]). These include high-performance liquid chromatography (HPLC), gas chromatography (GC), gas chromatography–mass spectrometry (GC–MS) and immunoassays, such as fluorescence polarization immunoassay (FPIA) [5] or enzyme-multiplied immunoassay (EMIT) [6]. Because of the lack of specificity of the immunoassays, HPLC is the method of choice for the simultaneous monitoring of CBZ and CBZ-E in plasma or serum samples. Several HPLC methods involving deproteinization [7], liquid–liquid extraction [8], solid–liquid extraction [9,10], column-switching [11,12] or on-line dialysis [13], combined with either isocratic or gradient elution, have been published. The analytes were detected in UV either at fixed low wavelengths [8–13] or by photodiode array detection [7]. Some procedures have been fully automated [12,13]. Most of the mentioned methods required special devices for the automation of the assay, tedious sample preparations or long chromatographic runs.

The drug assays performed by central laboratories in Phase II and III clinical trials require very fast turn-around times. Recently, the use of non-porous micropellicular column packings combined with modified HPLC systems allowed fast HPLC separations in a few seconds [14]. In this paper, we have applied this type of column to the assay of CBZ and CBZ-E. As a prerequisite, no modification of a regular HPLC system was allowed. The developed method and the validation data are described.

2. Experimental

2.1. Materials

CBZ, CBZ-E and 10,11-dihydro-*trans*-dihydroxycarbamazepine (CBZ-DIOL) were kindly provided by Ciba Geigy (Basel, Switzerland). Primidone and phenylethylmalonamide (PEMA) were kindly provided by ICI-Pharma (Luzern, Switzerland). Phenytoin free acid, (\pm)-5-(*p*-hydroxyphenyl)-5-phenylhydantoin (*p*-HPPH), 5-(*m*-hydroxyphenyl)-5-phenylhydantoin (*m*-HPPH), phenobarbital and ethosuximide were purchased from Sigma (St. Louis,

MO, USA). Felbamate suspension was supplied by Essex Chemie (Luzern, Switzerland). Vigabatrin was kindly provided by Marion Merrell Dow Research Institute (Cincinnati, OH, USA). Pheneturide was kindly supplied by Laboratoires Sauter (Vernier/Geneva, Switzerland). Clobazam and its metabolite, desmethylclobazam, were kindly supplied by Roussel Uclaf (Paris, France).

Acetonitrile and ethanol were of HPLC grade from Rathburn (Walkerburn, UK). Water was purified by a Milli Q Plus device from Millipore (Bedford, MA, USA). Potassium dihydrogenphosphate trihydrate and orthophosphoric acid (85%) were of analytical grade from Merck (Darmstadt, Germany).

Blank plasma and serum from healthy volunteers and plasma samples from patients receiving AEDs were used.

2.2. HPLC system

An HPLC system consisting of a Model 616 solvent delivery pump (Waters, Milford, MA, USA), a Waters Model 600S controller, a Model 717plus autosampler equipped with a temperature-controlled rack (Waters), a variable-wavelength UV detector from Shimadzu (Model SPD10-A; Burkard Instrumente, Zürich, Switzerland) and a Waters Maxima 825 data station were used. AEDs were separated on a Kovalis MS C₁₄ column (33×4.6 mm I.D., particle size 1.5 μ m; CU Chemie Uetikon, Uetikon, Switzerland). The column was immersed in a water bath operated at 60°C.

2.3. Assay conditions

The mobile phase was 0.02 M potassium phosphate buffer pH 7.0–acetonitrile (93:7, v/v). The flow-rate was 1.2 ml/min. Absorbance was monitored at 210 nm. The injected volume was 2 μ l.

2.4. Standard solutions

Stock and working solutions of AEDs were prepared in ethanol. All solutions were stable when stored at 2°C for several months [8].

2.5. Sample preparation

The plasma samples to be assayed (500 μl) were placed in the autosampler vials. Acetonitrile (850 μl) was added and the vials were vortex-mixed for a few seconds. The centrifuged vials (1500 $g/5$ min/25°C) were ready for injection.

2.6. Calibration curves

Up to six blank plasma samples were spiked with various amounts of the appropriately diluted reference solution. Standard samples were then prepared as the unknown samples. For standard samples, the volume of acetonitrile to be added corresponded to the regular volume to be added (850 μl) minus the volume of the working solution added.

The CBZ and CBZ-E concentrations were selected to cover the therapeutic ranges. Calibration curves were obtained by plotting the peak heights versus the nominal concentrations. The equations were calculated by the least-squares method using linear regression.

2.7. Method validation

The precision and accuracy of the method were evaluated by repetitive analysis of plasma samples ($n=10$) spiked with low, medium and high levels of CBZ and CBZ-E. Intra-day precision and accuracy data were obtained by the analysis of these samples on one day by the same operator. Inter-day precision and accuracy were obtained by assaying these three levels ($n=26$) on different days by two operators.

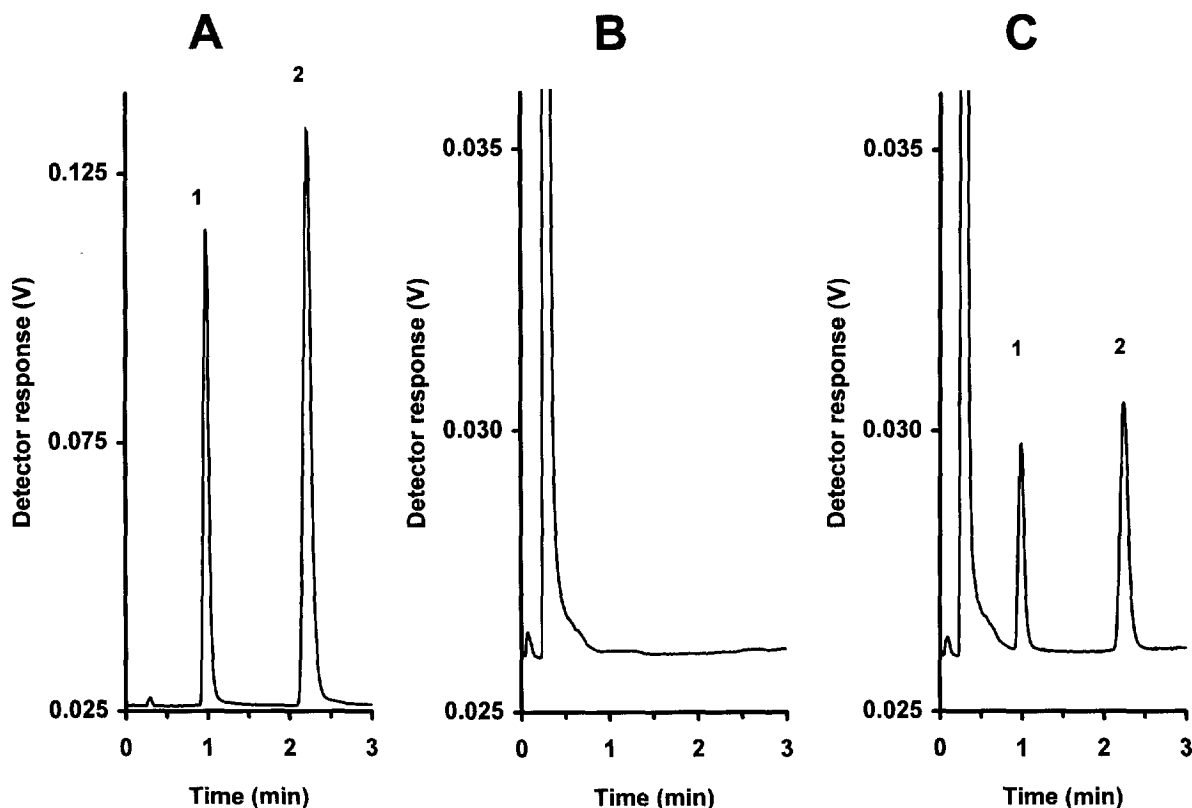


Fig. 1. Typical chromatograms of a working solution containing CBZ-E and CBZ (A), a blank plasma sample (B), a medium-range plasma standard spiked with 25.4 and 11.8 $\mu\text{mol/l}$ of CBZ and CBZ-E, respectively (C), a serum sample spiked with 45.1 $\mu\text{mol/l}$ of CBZ and 20.8 $\mu\text{mol/l}$ of CBZ-E used as quality control *high* (D) and a plasma sample collected from a epileptic patient under polytherapy (E). Peaks: 1=CBZ-E, 2=CBZ.

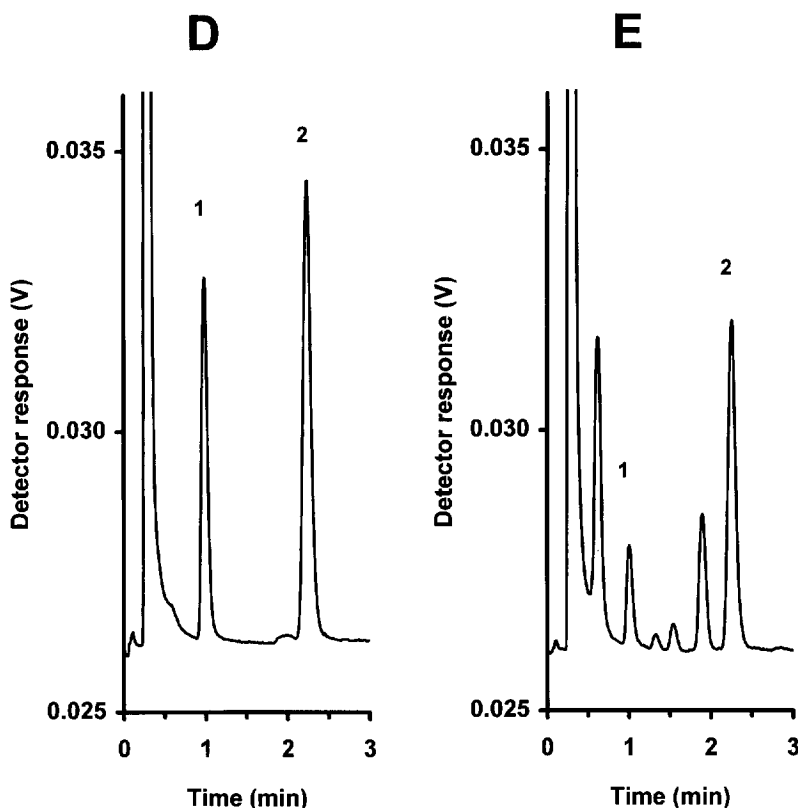


Fig. 1. (continued)

The recoveries were calculated by comparison of plasma samples ($n=9$) and aqueous samples ($n=3$). The linearity data were obtained by means of calibration curves ($n=30$). The ruggedness of the method was tested by its ability to allow the analysis of smaller volumes of sample than recommended by decreasing proportionally the acetonitrile volume to be added, and its ability to analyze serum samples instead of plasma samples.

3. Results and discussion

3.1. Chromatography

As a rule, rapid HPLC requires specially designed instrumentation having a low overall system dead volume, temperature control over a wide super-ambient temperature range, a precise sample introduction device with low dispersion, a highly sensi-

tive detector with short response time and a small flow-cell volume [14]. As a prerequisite of this work, no modification of a conventional HPLC system was applied. The commercially available micropellicular column, Kovasil MS C_{14} , was just installed instead of a regular C_{18} column.

Under the described conditions, CBZ and CBZ-E are well separated from nine other commonly prescribed AEDs and their metabolites in less than 2.5 min. The retention times were 2.27 ± 0.03 min (R.S.D., 1.3%; $n=40$) and 1.01 ± 0.01 min (R.S.D., 1.3%; $n=40$). Typical chromatograms of a working solution, a plasma standard, a quality control serum sample and a patient's sample are shown in Fig. 1. The relative retentions of commonly prescribed AEDs are given in Table 1. No endogenous compound was found to interfere in drug-free plasma samples collected from ten different volunteers. Due to the very low specific surface area of the packing, the organic modifier content in the mobile phase was

Table 1
Relative retention (*r*) of commonly prescribed AEDs

Drug	<i>t_R</i> (min)	<i>r</i> _{1, s=CBZ-E}
Valproic acid	N.D.	—
Vigabatrin	N.D.	—
Ethosuximide	0.31	0.08
PEMA	0.32	0.09
Primidone	0.40	0.20
Phenobarbital	0.40	0.20
Felbamate	0.43	0.24
<i>p</i> -HPPH	0.55	0.39
CBZ-DIOL	0.63	0.50
<i>m</i> -HPPH	0.63	0.50
CBZ-E	1.01	1.00
Pheneturide	1.21	1.26
Phenytoin	1.33	1.42
CBZ	2.27	2.66
Desmethyloclobazam	3.04	3.67
Clobazam	5.44	6.83

N.D.=not detected. For other abbreviations, see text.

decreased from about 30% for a conventional C₁₈ column to 7%. Taking into account both the time required for a chromatographic run and the amount of organic modifier required for the elution of CBZ, the acetonitrile consumption is reduced from about 6 ml to 0.25 ml per chromatographic run. The usual pressure was 2200 p.s.i. under the recommended experimental conditions. An increase of injected volumes to 5 μl lead to an unsatisfactory peak

broadening. Because of external standard calibration, the autosampler had to be able to inject volumes as low as 2 μl with good reproducibility. No problems were encountered with possible protein flakes floating at the top of the acetonitrile extract. The equilibration time was a few minutes, due to the rapid mass transfer of micropellicular packings. Several hundred injections were performed without degradation of the column's characteristics. The recommended method can be easily adapted to the monitoring of other specific AEDs.

Additional investigations showed that both the separation and the peak shape were improved and that the run time was shortened by decreasing the dead volumes of the regular HPLC system and by increasing the temperature.

3.2. Precision and accuracy

The precision and accuracy data are shown in Table 2. The intra-day coefficients of variation were found to be within 2.9 and 3.6% for CBZ and CBZ-E, respectively. The inter-day coefficients of variation were 3.6 and 6.8% at the lowest levels, respectively. The accuracy, which was expressed as the mean deviation, ranged from -1.3 to 3.6% and from -1.0 to 4.7%, respectively.

Table 2
Precision and accuracy of the CBZ and CBZ-E assays

Compound	Nominal concentration (μmol/l)	Concentration found (mean ± S.D.) (μmol/l)	R.S.D. (%)	Confidence interval of the mean value (P=95%) (μmol/l)	Accuracy (%)
<i>Intra-assay variability (n=10)</i>					
CBZ	4.23	4.30 ± 0.12	2.9	4.30 ± 0.09	1.6
	25.38	25.27 ± 0.41	1.6	25.27 ± 0.29	-0.4
	50.76	50.82 ± 1.03	2.0	50.82 ± 0.74	0.1
CBZ-E	1.97	2.02 ± 0.07	3.6	2.02 ± 0.05	2.9
	11.79	11.70 ± 0.13	1.1	11.70 ± 0.09	-0.7
	23.58	23.63 ± 0.34	1.4	23.63 ± 0.24	0.2
<i>Inter-assay variability (n=26)</i>					
CBZ	4.23	4.39 ± 0.18	4.2	4.39 ± 0.07	3.6
	25.38	25.06 ± 0.60	2.4	25.06 ± 0.24	-1.3
	50.76	50.77 ± 0.99	1.9	50.77 ± 0.40	0.0
CBZ-E	1.97	2.07 ± 0.14	6.8	2.07 ± 0.06	4.7
	11.79	11.67 ± 0.21	1.8	11.67 ± 0.08	-1.0
	23.58	23.62 ± 0.30	1.3	23.62 ± 0.12	0.2

The accuracy was defined as the percentage deviation between the mean concentration found and the theoretical concentration.

Table 3
Recovery

Compound	Nominal concentration ($\mu\text{mol/l}$)	Recovery (mean \pm S.D.) (%)
CBZ	4.23	99.6 \pm 1.8
	25.38	100.8 \pm 1.7
	50.76	102.3 \pm 2.2
CBZ-E	1.97	110.1 \pm 1.8
	11.79	102.4 \pm 1.3
	23.58	103.3 \pm 1.5

3.3. Calibration

Calibration curves in plasma were linear over the range 0 to 51 $\mu\text{mol/l}$ (12.0 $\mu\text{g/ml}$) and 0 to 24 $\mu\text{mol/l}$ (6.0 $\mu\text{g/ml}$) for CBZ and CBZ-E, respectively. The correlation coefficients calculated ranged from 0.9984 to 1.0000 (mean 0.9998, $n=30$) and from 0.9989 to 1.0000 (mean 0.9998, $n=30$) for CBZ and CBZ-E, respectively. The assay has the necessary sensitivity and linearity to cover the therapeutic range of CBZ and CBZ-E. The limit of quantification for CBZ and CBZ-E were found to be 4 and 2 $\mu\text{mol/l}$, respectively.

3.4. Recovery

The plasma samples were deproteinized with acetonitrile. The mean recoveries within the calibrated range were found to be quantitative (Table 3).

3.5. Ruggedness

Assays were performed with plasma volumes as low as 100 μl , in order to demonstrate the possible use of small plasma volumes. The results in Table 4 show that 100 μl plasma samples could be assayed with satisfactory precision and accuracy.

As shown in Table 4, serum samples might be used instead of plasma samples. In this case, a positive bias of +10% was detected for CBZ-E at the lowest concentration.

4. Conclusion

The developed method is well suited for the assay of CBZ and CBZ-E in clinical trials. CBZ and CBZ-E are separated from other concomitant medication in less than 2.5 min. No modification of a regular HPLC system was required.

Table 4

Ruggedness. The accuracy was defined as the percentage deviation between the mean concentration found and the theoretical concentration

Compound	Nominal concentration ($\mu\text{mol/l}$)	Concentration found (mean \pm S.D.) ($\mu\text{mol/l}$)	R.S.D. (%)	Confidence interval of the mean value ($P=95\%$) ($\mu\text{mol/l}$)	Accuracy (%)
<i>Plasma sample volume of 500 μl ($n=3$)</i>					
CBZ	8.50	8.75 \pm 0.16	1.8	8.75 \pm 0.39	3.0
	45.10	45.28 \pm 1.93	4.3	45.28 \pm 4.80	0.4
CBZ-E	3.90	3.89 \pm 0.05	1.3	3.89 \pm 0.12	-0.3
	20.80	20.41 \pm 0.51	2.5	20.41 \pm 1.26	-1.9
<i>Plasma sample volume 100 μl ($n=9$)</i>					
CBZ	8.50	8.88 \pm 0.15	1.7	8.88 \pm 0.12	4.4
	45.10	48.01 \pm 1.94	4.0	48.01 \pm 1.49	6.5
CBZ-E	3.90	3.95 \pm 0.08	1.9	3.95 \pm 0.06	1.3
	20.80	21.16 \pm 0.32	1.5	21.16 \pm 0.24	1.7
<i>Serum samples ($n=10$) (for corresponding plasma results see Table 2)</i>					
CBZ	4.23	4.43 \pm 0.11	2.5	4.43 \pm 0.08	4.8
	25.38	23.73 \pm 0.60	2.5	23.73 \pm 0.43	-6.5
	50.76	50.73 \pm 0.28	0.6	50.73 \pm 0.20	-0.1
CBZ-E	1.97	2.18 \pm 0.03	1.5	2.18 \pm 0.02	10.8
	11.79	11.21 \pm 0.27	2.4	11.21 \pm 0.19	-4.9
	23.58	23.60 \pm 0.17	0.7	23.60 \pm 0.13	0.1

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