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Micellar electrokinetic capillary chromatography for therapeutic drug monitoring of carbamazepine and its main metabolites

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Abstract

In carbamazepine (CBZ) therapy the concomitant monitoring of concentrations of CBZ and its metabolites is strictly recommended, primarily to avoid toxic side effects. Currently, clinical routine monitoring of CBZ is accomplished by high-performance liquid chromatography or immunological methods. In this study a micellar electrokinetic capillary chromatographic (MECC) method was developed for routine drug monitoring of CBZ and its main metabolites, carbamazepine 10,11-diol and carbamazepine 10,11-epoxide, in human serum or plasma samples. The MECC method enabled baseline separation of all analytes within 2.5 min. The assay revealed sufficient precision and sensitivity and the results of either an automated HPLC or the MECC chromatography assay were in good agreement ($r = \geq 0.97$). The maximum deviation for CBZ was 0.26 μM.

Keywords: Carbamazepine

1. Introduction

Carbamazepine (CBZ) is considered as a first line drug in the treatment of most forms of epilepsia [1,2], and has recently gained further interest for its mood stabilizing properties [3–5]. In contrast to tricyclic antidepressants where the value of therapeutic drug monitoring (TDM) is still a matter of debate, concomitant TDM is regarded as indispensable in the therapy with CBZ [6–8].

To avoid toxicity and achieve optimal clinical response a therapeutic plasma level range of CBZ between 17 and 51 μM has been widely accepted [9]. Similar to other tricyclic CNS drugs, CBZ is a highly lipophilic drug which undergoes an extensive hepatic phase I metabolism with the CBZ-epoxide (CBZE) and CBZ-diol (CBZD) formation accounting for about 80% of the total drug clearance in steady state [10,11].

It is therefore suggested that only the determination of CBZ together with its main metabolites reveals sufficient information to optimize individual CBZ-dosages. This is particularly true for CBZE which by itself exerts anticonvulsant effects but also neurotoxicity [12,13]. Moreover, only the determination of CBZ and its main metabolites provides detailed information on possible pharmacokinetic

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interactions of CBZ with concomitantly administered drugs.

For routine clinical monitoring either immunological or chromatographic methods are currently available [14–16]. However, immunological methods are often subject to problems with cross-reactive interferences and no immunological method can, as yet, distinguish between CBZ, CBZE and CBZD [17]. High-performance liquid chromatography (HPLC), still used as standard reference method, suffers mainly from its extensive use of expensive and polluting organic solvents [18].

The application of capillary electrophoresis (CE) was broadened by the introduction of the micellar electrokinetic capillary chromatography (MECC). MECC has been found suitable for the determination of a growing number of drugs in a TDM setting [19–21]. Anticonvulsants including CBZ were one of the first groups of drugs analyzed by the MECC technique [22–25], but none of these reports included the concomitant determination of the metabolites.

The main purpose of this report is to describe MECC combined with photodiode-array detection as an alternative approach for the qualitative and quantitative analysis of CBZ and its two main metabolites in serum, CBZE and CBZD. We have therefore compared the MECC results with those obtained by an established HPLC method including on-line sample extraction.

2. Experimental

2.1. Patients

This study included 15 hospitalized patients suffering from either epilepsia, depression or mania. Blood samples were taken for routine monitoring at least 7 days after start of CBZ therapy to reach steady state conditions. All procedures were in accordance with the ethical standards of the Helsinki declaration.

2.2. Chemicals

CBZ and its epoxide- and diol-metabolites were gifts from Novartis (Basel, Switzerland). Methylpropylsuccinimide (MPS) used as internal standard (I.S.) was purchased from Sigma (St. Louis, MO, USA). Deionized water was processed by a Milli-Q water purification system (Millipore, Eschborn, Germany). All other chemicals were of analytical grade and purchased from Merck (Merck, Darmstadt, Germany).

2.3. Drug standards

Stock solutions of the analytes and the internal standard were prepared by dilution in methanol and kept at −20°C for up to 3 months. Working standards were prepared by supplementing blank plasma with diluted stock standards to reach concentrations standards in a range between 2.5 and 37.1 μ*M* for CBZ, 0.4 and 5.9 μ*M* for CBZE and 0.79 and 11.8 μ*M* for CBZD. The I.S., MPS, was used at a concentration of 120 μ*M*.

2.4. CE instrumentation and MECC operating conditions

A HPCE 3D capillary electrophoretic system equipped with a diode-array UV absorbance detector (Hewlett-Packard, Waldbronn, Germany) was used. The detector was routinely set to 210 nm. The capillary was unmodified silica (Microquartz, Munich, Germany) 34 cm (effective length 25.5 cm)×50 μm I.D. The running buffer consisted of 0.025 *M* $Na_2B_4O_7$ (pH 9.2) and 0.05 *M* sodium dodecylsulfate (SDS).

Prior to each injection, the capillary was rinsed for 2 min with 0.1 *M* sodium hydroxide and 5 min with running buffer. The capillary temperature was kept constant at 30°C and a voltage of +25 kV was applied. Samples were injected hydrodynamically with 5000 Pa for 3 s.

Peaks in serum samples of patients treated with CBZ could be identified by comparison of the migration time and by comparison of UV spectra recorded between 190 and 350 nm with those of the previously investigated control standards. As shown in Fig. 1, identical UV spectra for both, a patient sample and a control standard, containing similar amounts of the analytes, at the same migration time, gave high evidence of identical analytes in both samples.

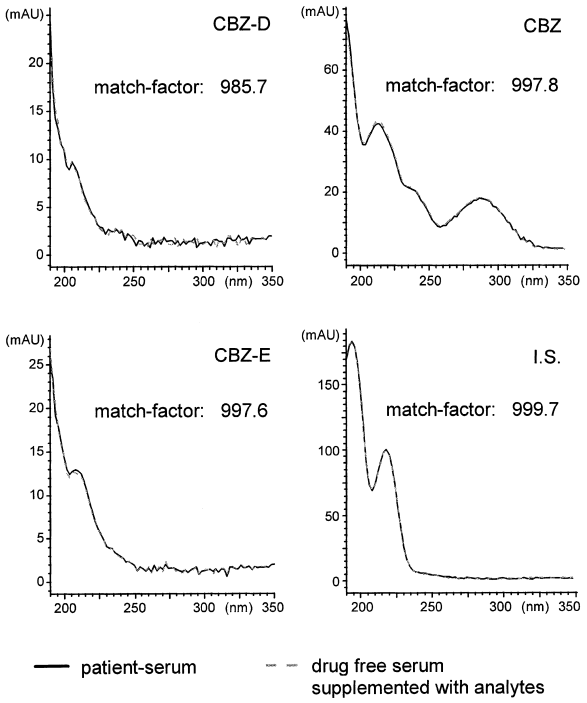

Fig. 1. Peak identification by comparison of UV spectra obtained after normalization and overlay of spectra from MECC analysis of drug free serum spiked with CBZ and metabolites (hatched line) and serum of a patient treated with CBZ (solid line). The match factor indicates the statistical probability of peak identity.

2.5. Sample pretreatment

One milliliter of serum or plasma samples was supplemented with the I.S. and extracted by a simple liquid–liquid extraction using 6 ml ethyl acetate. After vortex mixing and centrifugation at 2000 *g* for 15 min, 4 ml of the organic layer were transferred to a polypropylene tube and evaporated to dryness under a gentle stream of N_2. Samples were reconstituted with 100 μl of 5% methanol in background electrolyte for injection onto the CE system.

2.6. HPLC instrumentation and operating conditions

The chromatographic system consisted of an Abimed autosampler 231 (Abimed, Langenfeld, Germany), an UV absorbance detector Spectro Monitor 3000 (TSP, Darmstad, Germany) set to 204 nm, a six-port switching valve H Besta (Besta, Heidelberg, Germany), a Varian 9010 gradient pump (Varian, Darmstadt, Germany) and a ConstaMetric III pump (TSP). The clean-up column 20×4.6 mm I.D. was filled with Hypersil ODS, 5-μm particle size (supplied by MZ-Analysentechnik, Mainz, Germany). The analytical column, a Hibar® column, 125×4 mm I.D., filled with LiChrospher RP-8 (5-μm particle size), was supplied by Merck.

Direct injection of plasma or serum samples was enabled by use of a column-switching system that was adapted from an established method [26]. In brief, serum- or plasma samples were supplemented with I.S. (MPS, 20 μg/ml), directly injected to the clean-up column and pretreated by flushing with 1 ml/min 0.02 *M* KH_2PO_4 for 8 min. After 8 min, the valve was switched to transfer the analytes to the analytical column and reset after 30 min to proceed to the next sample. Chromatographic separation was achieved by gradient elution starting with 85% (v/v) eluent A (0.02 *M* KH_2PO_4 buffer) and 15% eluent B [(0.02 *M* KH_2PO_4 buffer–acetonitrile, 1:1, v/v)] and ending with 5% eluent A and 95% eluent B after 41 min. The flow-rate was 1.0 ml/min.

3. Results

Separation of CBZ and its metabolites was achieved by MECC in less than 2.5 min with baseline separation of all analytes, as shown in Fig. 2B Fig. 3. No interferences with either endogenous compounds or comedication were detectable as shown in the electropherogram obtained after analysis of serum of a patient treated with CBZ (Fig. 3).

Migration times were stable under the chosen conditions with a maximum variability of ±1.3% for CBZ and CBZE, ±1.1% for CBZD, and ±0.6% for the I.S. (n=95, on 10 different days). Moreover, changing the capillaries — even if we replaced the capillary by a capillary from another supplier — resulted in maximal deviations in the migration time of ≤0.1 min and no quantifiable deviations in peak areas or peak heights.

3.1. Recovery, linearity, and precision of the MECC analysis

Overall recovery was calculated by regression analysis from the comparison of calibration curves obtained from either the analysis of nonextracted

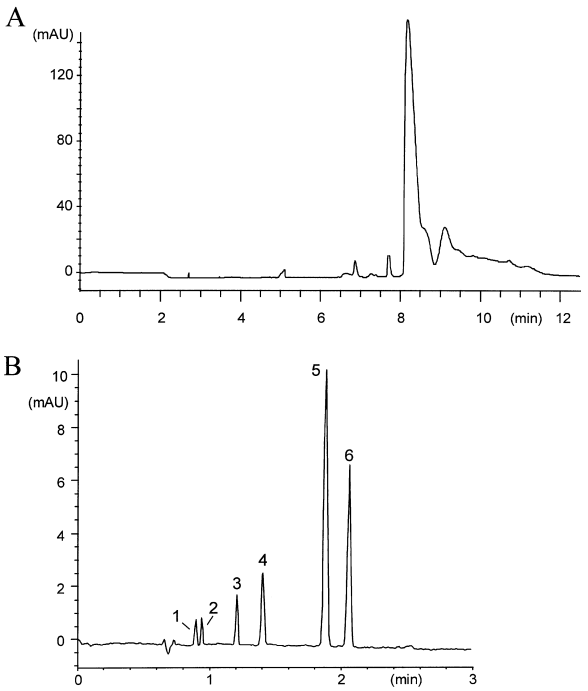

Fig. 2. Typical electropherograms obtained from the analysis of blank serum (A) and a standard mix of four different anticonvulsant drugs and carbamazepine metabolites (B). 1=Ethosuccimide (5 μ*M*), 2=primidone (5 μ*M*), 3=phenytoin (5 μ*M*), 4=CBZD (5 μ*M*), 3=CBZE (10 μ*M*), 4=CBZ (10 μ*M*). Buffer: 0.025 *M* $Na_2B_4O_7$ (pH 9.2); 0.05 *M* SDS; injection: 5000 Pa/3 s.; capillary 34/25.5 cm× 50 μm I.D., detection: UV, 210 nm; voltage: *V*=25 kV (*I*=140 μA, 30°C); *x*- and *y*- axis are different for electropherograms A and B.

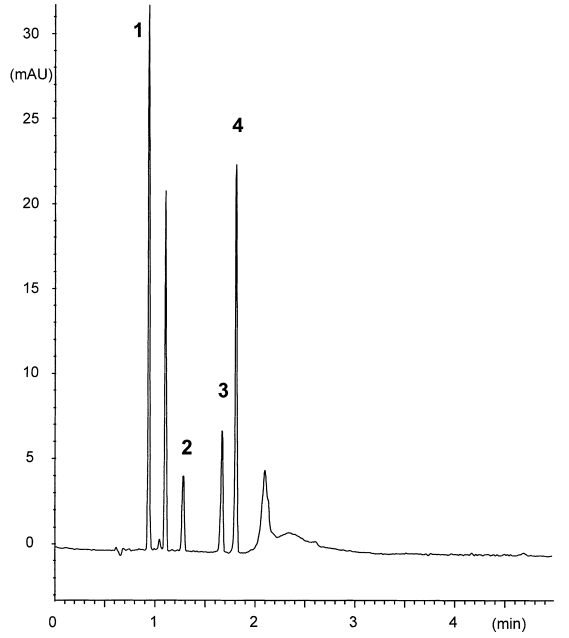

Fig. 3. Typical electropherogram obtained from the analysis of serum of a patient treated with CBZ. 1=I.S., 2=CBZD (7.5 μ*M*), 3=CBZE (5.9 μ*M*), 4=CBZ (29.6 μ*M*). buffer: 0.025 *M* $Na_2B_4O_7$ (pH 9.2); 0.05 *M* SDS; injection: 5000 Pa/3 s.; capillary 34/25.5 cm× 50 μm I.D., detection: UV, 210 nm; voltage: *V*=25 kV (*I*=140 μA, 30°C).

standard solutions or the analysis of spiked serum or plasma samples after extraction. The concentration of the extracted samples was calculated by means of the calibration curve obtained from the analysis of nonextracted standard samples. The recovery of CBZ and metabolites is given in Table 1.

Reproducibility calculated from the analysis of control samples on 5 different days gave maximum relative standard deviations (R.S.D.s) of ±8.4% for CBZ, ±14.4% for CBZE, and ±7% for CBZD, maximum inaccuracy was +1.3%, −2.5%, and −6%, respectively.

The detection limit, calculated by the method of Luthardt et al. [27], was found to be 0.5 μ*M* for CBZ, 0.03 μ*M* for CBZD and 0.1 μ*M* for CBZE. No interferences were detectable from other psychotropic drugs (antidepressants, neuroleptics or tranquillizers) within their particular therapeutic concentrations. Other anticonvulsants like phenytoin, ethosuccimide and primidone were sufficiently separated from CBZ and its metabolites as shown in Fig. 2B.

3.2. Precision of the HPLC method

The HPLC method revealed recoveries which were always >90% for all analytes at three different concentration levels (low, medium, and high). The day-to-day R.S.D. determined on at least 20 different days ranged between ±2.5 and 3.6% for CBZ, ±2.7 and 3.6% for CBZD, and ±2.3 and 4.0% for CBZE.

3.3. Comparison between HPLC and MECC

Based on a linear regression analysis of the concentrations detected with each method we found an extremely high convergence of the results as shown in Table 2.

Table 1
Recovery, inter-day and intra-day reproducibility for the determination of carbamazepine (CBZ), carbamazepine 10, 11-diol (CBZD), and carbamazepine 10,11-epoxide (CBZE) by MECC

Analyte	Reproducibility						Recovery (%) (% error)
	Inter-day (n=5)			Intra-day (n=9)			
	Conc. given (μM)	Conc. found (μM)	R.S.D. (%)	Conc. given (μM)	Conc. found (μM)	R.S.D. (%)	
CBZ	9.8	9.9	8.4	14.7	14.1	0.56	100 (7.6)
CBZD	2.9	2.7	7.0	3.6	3.55	0.54	93.2 (13)
CBZE	1.6	1.5	14	2	1.94	1.6	83.4 (14)

R.S.D. = relative standard deviation
Recovery was assessed by comparison (n=3) of calibration curves (five different concentrations) of either nonextracted standard samples or spiked serum samples after liquid–liquid extraction.

4. Discussion

Within this study, samples were analyzed both by MECC and an established HPLC method. Both methods gave similar concentrations of CBZ, CBZD and CBZE. The greatest deviation between the two methods was found for CBZ with a mean difference of 0.26 ± 2.9 μM.

Although the number of samples was rather limited, the MECC method showed sufficient precision and linearity to possibly serve as method for routine TDM. The limit of detection, which is normally a crucial point for CE in a TDM setting, was found to enable the quantification of all analytes within a therapeutically relevant range.

The separation efficiency of the MECC in comparison to HPLC-techniques was much higher with a calculated number of theoretical plates of 94 000 for CBZD and 427 000 for CBZ ($N \times m^{-1}$) and allowed baseline separation of all analytes within 2.5 min. Although a very fast analysis could be achieved by MECC, a drawback compared to the automated HPLC analysis was the requirement for a previous extraction procedure to remove matrix components. MECC analyses with direct plasma sample injection have been reported recently [19,24]. However, our efforts to inject plasma or serum samples directly were unsuccessful since endogenous compounds interfered with the analytes. The simple liquid–liquid extraction used in this first approach may be replaced by solid-phase extraction which can be easily automated.

Apart from the sample pretreatment, the operating costs are in general much lower and the separation is much faster for the developed MECC method. Moreover, little or no expensive and polluting organic solvents are required for MECC.

In conclusion, we have developed a very fast and simple MECC method for the determination of CBZ and its main metabolites. MECC has proven to be a useful or even advantageous alternative to HPLC for monitoring of CBZ and its main metabolites. Since the metabolites, CBZE and CBZD, are also detectable by this method, it should enable studies of CBZ metabolism and pharmacokinetic drug interactions.

Table 2
Comparison between the concentrations of carbamazepine (CBZ), carbamazepine-10,11-diol (CBZD), and carbamazepine 10,11-epoxide (CBZE) found after HPLC or MECC analysis

Analyte	n	Slope	Intercept	Correlation coefficient (r)
CBZ	15	1.07	−0.041	0.969
CBZD	9	1.09	−0.09	0.987
CBZE	8	0.92	0.07	0.988

Evaluation of convergence based on a linear regression analysis.

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