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High-performance liquid chromatographic determination of carbamazepine and metabolites in human hair

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Abstract

To study the use of hair analysis in monitoring drug compliance and historical changes in pharmacokinetics we developed a method for the quantitative determination of the anti-epileptic drug carbamazepine (CBZ) and *trans*-10,11-dihydro-10,11-dihydroxy-carbamazepine (CBZ-diol) in hair from carbamazepine users. Digestion by 1 *M* NaOH was found to be the best method for isolating CBZ and CBZ-diol from hair, followed by solid-phase extraction and reversed-phase HPLC with UV detection. Recoveries from spiked hair samples were 76–86%. Within-day precision (C.V.; $n=10$) for CBZ and CBZ-diol in hair of a CBZ user containing 10.9 μg/g CBZ and 3.2 μg/g CBZ-diol were 1.7 and 5.0%, respectively. Sectional hair analysis of a patient on a constant dosage of CBZ demonstrates an exponential decrease in hair concentrations of CBZ and CBZ-diol with increasing distance from the root, probably caused by shampooing. No CBZ-10,11-epoxide (CBZ-epox) could be detected. However, one component in the chromatogram is probably CBZ-β-hydroxythioether, an adduct of CBZ-epox with cysteine, or acridinethioacetal, its rearrangement product. The concentration of this component does not decrease with increasing distance from the root.

Keywords: Hair; Carbamazepine; *trans*-10,11-Dihydro-10,11-dihydroxycarbamazepine

1. Introduction

According to several studies, hair analysis seems promising for monitoring historical drug abuse [1–5], prenatal exposure to drugs [6,7], smoking habits [6,8,9] and compliance of medication intake [10–13]. It is generally assumed that drugs enter hair by passive diffusion from the circulatory system to growing cells at the base of the hair follicles and are encapsulated in keratin fibres of the hair shaft. More recent experiments suggest multiple mechanisms, such as transfer of drugs to hair from deep skin compartments and via sweat and sebum. Human scalp hair from the vertex region grows at an average rate of 1.3 cm/month. By analyzing various hair segments at different distances from the root, it is possible to reconstruct the time course of drug consumption [1,14].

Different kinds of techniques to isolate drugs from hair are used, such as acid extraction, digestion by NaOH, enzymatic hydrolysis and supercritical fluid extraction. Purification is performed by solid-phase extraction or liquid–liquid extraction. Determination of the drugs is carried out by GC–MS, RIA or HPLC with UV or fluorescence detection.

Carbamazepine (CBZ) is one of the most fre-

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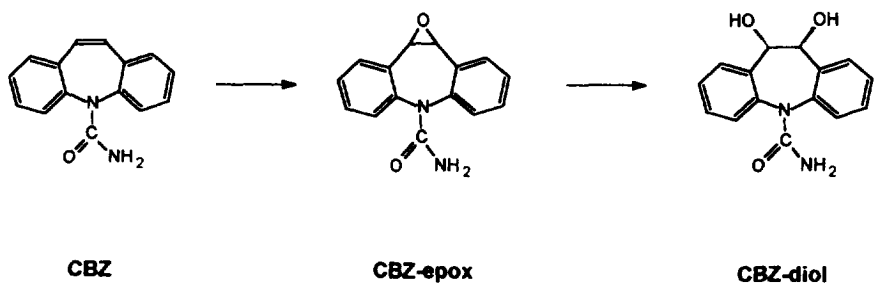

Fig. 1. Main metabolic pathway of carbamazepine.

quently used anti-epileptic drugs for treatment of complex partial, elementary partial and generalized tonic-clonic seizures. Its main metabolic pathway (Fig. 1) is epoxidation to carbamazepine-10,11-epoxide (CBZ-epox), followed by conversion to *trans*-10,11-dihydro-10,11-dihydroxy-carbamazepine (CBZ-diol).

At least a third of epileptic patients whom are prescribed anti-epileptic drugs do not have a reliable drug intake. Because non-compliance can result in poor seizure control, it is important to assess compliance by determination of drug serum levels, level/dose ratio, intraindividual variability and by patient self-report. These methods all have limited value [15]. We intend to evaluate the value of hair analysis of CBZ, especially as a supplement to common methods in monitoring drug compliance. Several chromatographic methods have been described for analysis of CBZ and metabolites in body fluids, but not in hair [16–18].

In this paper we describe the development and validation of an HPLC method for the measurement of CBZ and CBZ-diol in human scalp hair.

2. Materials and methods

2.1. Solvents, chemicals and standards

Methanol (Lichrosolv), acetone (Uvasol), ethylacetate (Lichrosolv), NaOH (p.a.), H_3PO_4 (p.a.), KH_2PO_4 (p.a.), K_2HPO_4 (p.a.), sodium acetate (p.a.) and HCl (p.a.) were obtained from Merck (Darmstadt, Germany), Supergradient grade acetonitrile was obtained from LabScan Analytical Sciences (Dublin, Ireland). C_{18} Solid-phase extraction columns (100 mg) were purchased from J.T. Baker (Philipsburg, NJ, USA). 5-Ethyl-5-*p*-tolyl-barbituric acid (gold label) (ETB) was obtained from Aldrich (Beerse, Belgium). CBZ, CBZ-diol and CBZ-epox were obtained from Ciba Geigy (Basel, Switzerland).

2.2. Preparation of standards

Stock solutions of CBZ, CBZ-diol, CBZ-epox and ETB (1.00 mg/ml) were prepared in methanol. A standard solution of ETB (25 μg/ml) was prepared in 0.2 *M* phosphate buffer (pH 2.1). Standard solutions of CBZ,CBZ-diol and CBZ-epox were prepared in methanol.

2.3. Subjects and samples

For the development of this method, hair samples were obtained from a number of patients from the Instituut voor Epilepsiebestrijding "Meer en Bosch" under anti-epileptic therapy with CBZ. A full-length hair sample from the posterior vertex region of one of these patients was used to monitor historical drug intake. This sample was cut into sections. We also obtained hair samples from eight volunteers without any medication to use both as blank hair, and as reference material after spiking with CBZ, CBZ-diol and CBZ-epox.

2.4. Final analytical procedure

2.4.1. Sample preparation and extraction

Hair samples of 20–50 mg are cut in fragments of 5 mm, weighed and washed with 5 ml of methanol for 15 min at 37°C once, and with 5 ml of water for

15 min at 37°C twice in order to remove external contamination and fat.

After decontamination of the hair samples, fixed amounts of CBZ, CBZ-diol and CBZ-epox are added to the blank hair samples in order to obtain suitable reference material.

Digestion is carried out in test tubes with 1 *M* NaOH by carefully shaking for 20 h at 37°C.

After digestion, the mixture is centrifuged for 10 min at 3000 rpm (1500 *g*). The supernatant is transferred to a test tube containing 0.75 ml 1 *M* H_3PO_4–KH_2PO_4 (4:1, v/v). After addition of 50 μl (25 μg/ml) internal standard (ETB) the sample is applied to a conditioned C_{18} solid-phase extraction column, followed by rinsing twice with 1 ml 0.1 *M* KH_2PO_4 and once with 1 ml of methanol–water (15:85, v/v). After drying the column, the fraction containing CBZ and metabolites is eluted with 0.6 ml ethylacetate. This extract is evaporated at 40°C for 40 min. The residue is dissolved in 200 μl methanol–water (25:75, v/v). A 100-μl sample is injected into the chromatograph.

2.4.2. HPLC

This method is developed using a liquid chromatograph (Perkin-Elmer series 4), an autosampler (Perkin-Elmer ISS-100), a scanning detector (TSP Spectra FOCUS), integrating software (TSP Spectra SYSTEM Software) and a Spherisorb 3 ODS-2 column (15×0.46 cm) from Phase Separations (Waddinxveen, Netherlands).

The mobile phase, which is deaerated with helium, consists of a mixture of acetonitrile, methanol and water. To adjust for changes in baseline during gradient analysis, 0.01 *M* sodium acetate is added to the mobile phase. The gradient is performed at room temperature and its composition during a run is shown in Table 1. Flow-rate is 1 ml/min.

The UV absorbance is monitored in a wavelength interval of 195–345 nm. Quantification is performed by measuring peak height at 205 nm (internal standard method, one-point calibration). Relative retention times are 0.71 and 1.16 for CBZ-diol and CBZ, respectively.

3. Results and discussion

We studied several digestion and extraction techniques to isolate CBZ and metabolites from decontaminated human scalp hair.

Digestion with NaOH was optimized for concentration (0.1–4.0 *M*), time (0.75–20 h) and temperature (37°C, 55°C and 70°C). Digestion with 1.0 *M* NaOH for 20 h at 37°C gave maximum recovery of CBZ and CBZ-diol. Lower concentrations of NaOH led to incomplete dissolution of the sample, higher concentrations to destruction of CBZ and CBZ-diol. Digestion at temperatures of 55°C and 70°C caused many interfering components in the chromatogram.

Extraction was carried out for 20 h by either methanol, methanol–5 *M* HCl (20:1, v/v), acetonitrile or acetone. The solvent was then evaporated and the residue was dissolved in 1 ml of 0.2 *M* phosphate buffer (pH 6.5), followed by solid-phase extraction. Extraction with methanol–5 *M* HCl gave the best results with clean chromatograms and was then optimized for time (1–20 h) and temperature. Maximum recovery was achieved after extraction for 20 h at 37°C. However, even after extraction under optimal conditions, approximately 40% of CBZ and CBZ-diol was not extractable and could only be isolated after digestion with NaOH. This percentage did not decrease when extraction was carried out repeatedly.

Because digestion of hair with NaOH gave higher

Table 1
Linear gradient composition

Duration (min)	From (%A:%B:%C:%D)	To (%A:%B:%C:%D)
0–18	0:25:4:71	18:25:0:57
18–26	18:25:0:57	18:25:0:57
26–31	18:25:0:57	0:25:4:71
31–	0:25:4:71	0:25:4:71

A=acetonitrile; B=methanol; C=0.01 *M* sodium acetate; D=water.

recovery of CBZ and CBZ-diol than extraction with methanol–5 *M* HCl, we preferred digestion to extraction. Recovery of digestion was not increased when hair samples were cut in fragments of 1 mm, instead of 5 mm. The large number of components seen in the chromatogram after digestion placed great demands on the chromatography. Reliable detection of CBZ and CBZ-diol required a gradient analysis as a result of which relatively polar components were delayed and interference of unknown substances with CBZ-diol could be prevented. Typical chromatograms of a "blank" hair sample of a volunteer without any medication and a hair sample of a CBZ user are shown in Figs. 2 and 3, respectively.

3.1. CBZ-epox

CBZ-epox can be determined with our method, but needs addition of 0.1 ml 30% H_2O_2 to 1 ml 1 *M* NaOH before digestion. When CBZ-epox is added to blank hair before digestion with NaOH, CBZ-epox cannot be detected. However, in combination with H_2O_2, CBZ-epox remains detectable in the chromatogram with a relative retention time of 0.86.

Neither digestion with 1 *M* NaOH in combination with H_2O_2 addition, nor extraction with methanol–HCl, yields detectable amounts of CBZ-epox in hair of a CBZ user.

Chromatograms of CBZ users show an extra peak with a relative retention time of 0.95, which cannot

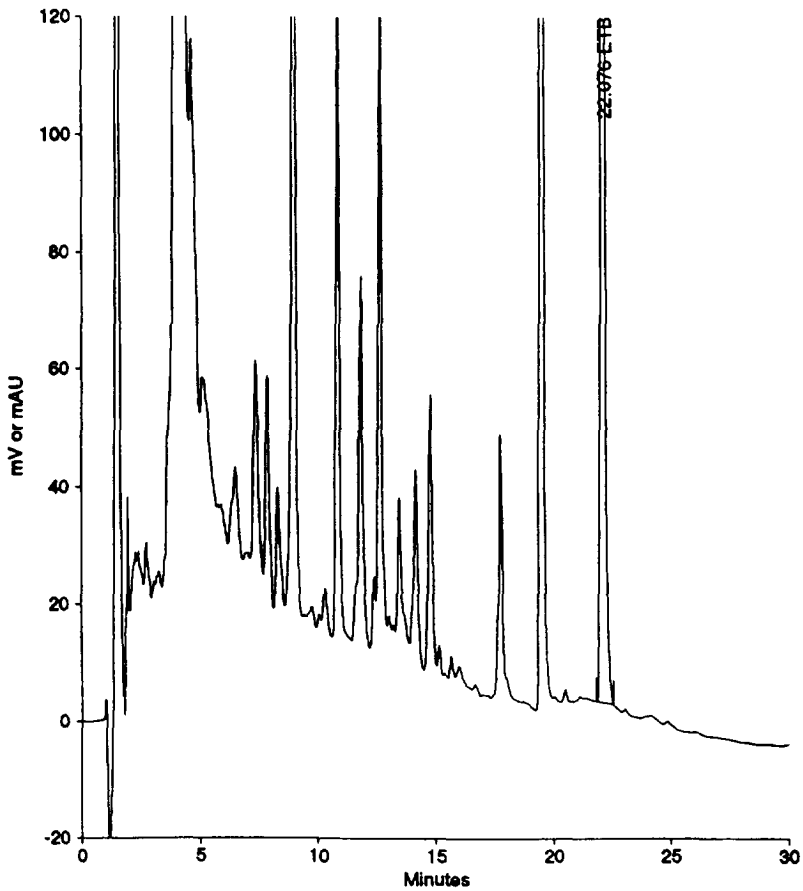

Fig. 2. Chromatogram of "blank" hair from a volunteer without any medication.

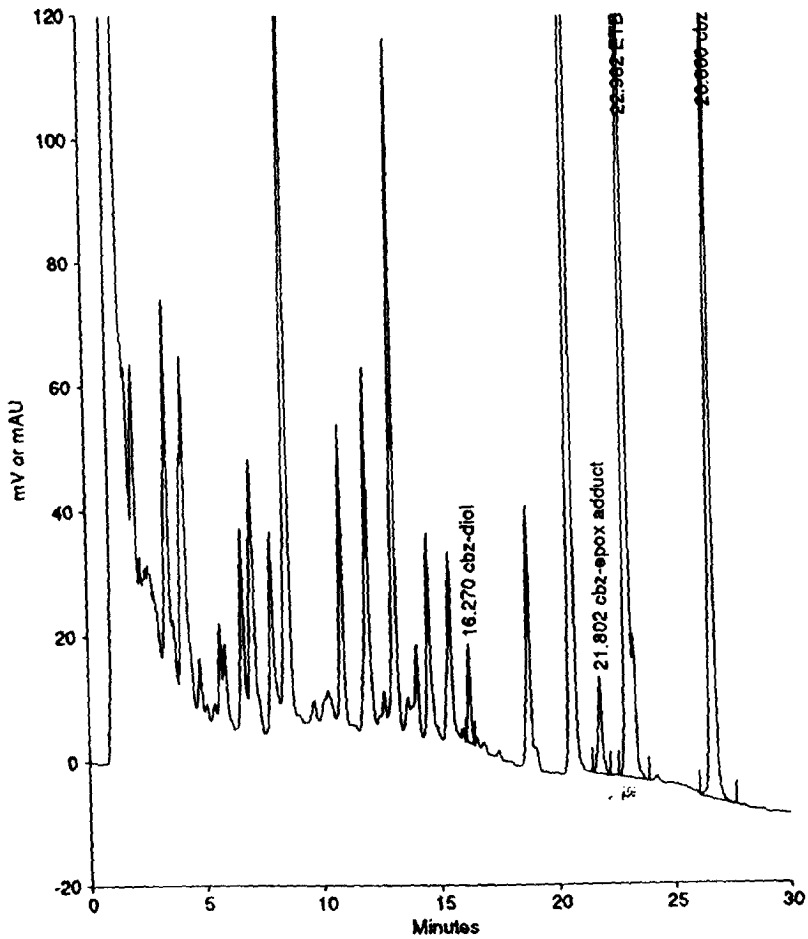

Fig. 3. Chromatogram of hair from a patient using CBZ.

be detected in blank hair from volunteers. A substance with the same retention time and UV spectrum can be produced in vitro by addition of CBZ-epox to blank hair or cysteine before digestion with NaOH. We postulate a nucleophilic addition of the thiol, resulting in the production of carbamazepine-β-hydroxythioether, possibly followed by rearrangement into an acridinethioacetal [19]. Because recovery of the in vitro reaction is very low, the substance found in hair of patients using CBZ is probably produced in vivo. Identification and synthesis of this CBZ-epoxide adduct will be performed. Till then, to estimate concentrations of the CBZ-epox adduct we measured relative peak-height ratios of the adduct in comparison with the internal standard and calculate a CBZ-epox adduct equivalent with the CBZ calibration.

3.2. Recovery, linearity and reproducibility

Recoveries after digestion by 1 *M* NaOH for 20 h at 37°C and solid-phase extraction of CBZ and CBZ-diol added to blank hair were determined at quantities of 0.25 and 4.00 μg ($n=6$), corresponding to 5 μg/g and 80 μg/g in a sample of 50 mg. Recoveries were found to be 86% and 84% at 0.25 μg and 83% and 76% at 4.00 μg for CBZ and CBZ-diol, respectively.

The calibration curves for added CBZ and CBZ-diol were linear in the range 0–4.00 μg.

The regression equations were $y=1.09x-0.49$ ($r=1.000$) for CBZ and $y=1.04x-0.23$ ($r=1.000$) for CBZ-diol (5 concentrations in triplo).

Reproducibility of the assay was calculated by replicate analysis of a homogenized hair sample of a patient using CBZ, containing 10.9 μg/g CBZ and 3.2 μg/g CBZ-diol. The within-day precision (C.V.; $n=10$) was 1.7% for CBZ and 5.0% for CBZ-diol.

The limit of quantitation was estimated at 25 ng (corresponding to 0.5 μg/g in a sample of 50 mg). When 25 ng was added to a blank hair sample, the C.V. was found to be 4.9% for CBZ ($n=9$) and 9.1% for CBZ-diol ($n=8$).

3.3. Case study

A 40 year-old woman with a reliable drug intake has used CBZ in a dose of 400 mg per day for over six years. A hair sample from the posterior vertex was cut into sections in which CBZ, CBZ-diol and CBZ-epox adduct equivalent were determined. These analyses were repeated in a second hair sample taken one year later. The results of these analyses are presented in Fig. 4. Firstly these curves illustrate that in spite of the constant dose, the concentrations of CBZ and CBZ-diol in hair decrease with increasing distance from the root. This decline follows an exponential curve. Half-life times in the first hair sample were 5.4 months for CBZ and 5.2 months for CBZ-diol. Secondly it shows that the concentration of CBZ-epox adduct equivalent does not decrease with increasing distance from the root. Finally, Fig. 4 demonstrates that the curves can be highly reproducible within a patient under the same drug regimen.

The last few years there have been a growing number of studies on testing human hair for drugs of abuse, which report that the drug levels in the sections towards the root were higher than in the sections nearest the tip [20]. These findings correspond to the decrease of drug levels in our hair samples of CBZ-users.

Towards the end of hair there is an increasing loss of cuticle cells due to weather and cosmetic treatments, such as brushing and combing [20]. The

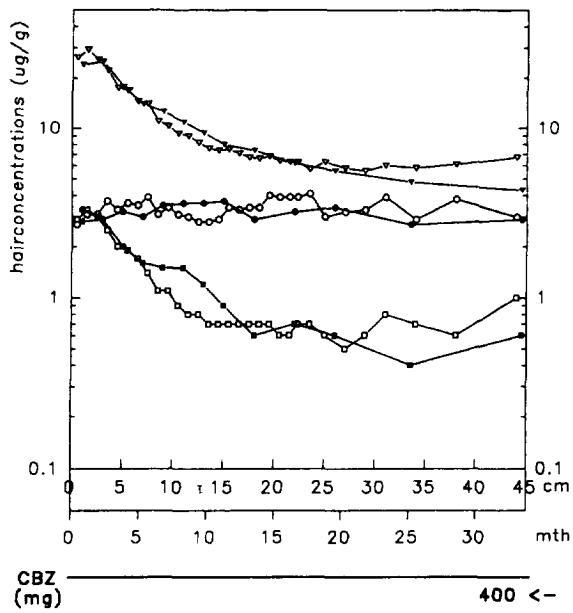

Fig. 4. Concentrations of CBZ (▼), CBZ-diol (■) and CBZ-epox adduct equivalent (●) related to distance from root and time before sampling (growth rate: 1.3 cm/month) obtained from the sectional analysis of two hair samples from a patient with a constant dose of 400 mg CBZ (filled symbols: 090395; hollow symbols: 070396).

cuticle forms a protective coating which encloses the hair fibre and acts as a chemical barrier. As a result of the damaged cuticle, the hair shaft becomes more sensitive to regularly shampooing [21,22] which may explain the gradual wash-out of CBZ and CBZ-diol from hair. The relatively constant amount of CBZ-epox adduct is probably due to a covalent binding of CBZ-epox to cysteine in hair, which is not sensitive to shampooing.

We developed a reliable method for the determination of CBZ and CBZ-diol in human scalp hair. The method is useful to detect and quantitate factors, which are important for drug incorporation into hair and wash-out from hair. Whether this method could provide information on historical drug consumption is under present study. Because both CBZ and CBZ-diol can be determined in hair, this method might also be useful when unexpected toxicity occurs as a result of changes in metabolism.

Acknowledgments

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