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Determination of carbamazepine and its main metabolite in human hair by capillary electrophoresis and liquid chromatography techniques, both coupled with mass spectrometry

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ABSTRACT

The aim of the research was to determine carbamazepine (CBZ) and its main metabolite 10,11-epoxy-10,11-dihydro-carbamazepine (CBZ-E) in human hair using the capillary electrophoresis (CE) system coupled with mass spectrometry detection (MS) and to compare the obtained results with the liquid chromatography (LC) technique, also coupled with the MS detector. Hair samples were prepared using microwave-assisted extraction (MAE) at 60 °C for 10 min in an alkaline solution (pH = 10) with ethyl acetate as the extraction solvent. In the frame of this study, the procedure for the separation of CBZ and CBZ-E using the CE-MS technique was developed. The best results were achieved using 10 mM ammonium acetate (pH = 6.8) as the background electrolyte (BGE), after filling the capillary with 1% highly sulfonated β-cyclodextrin (HSβCD) in 10 mM ammonium acetate. Then, the validation parameters of the MAE/CE-MS and MAE/LC-MS methods such as: limit of detection (for CBZ are: 0.36 and 0.22 ng/mg, respectively; for CBZ-E 0.38 and 0.17 ng/mg, respectively), limit of quantification (for CBZ are: 0.86 and 0.72 ng/mg, respectively; for CBZ-E 0.94 and 0.56 ng/mg, respectively), precision (6.91–14.5% and 2.16–15.6%, respectively), recovery (87.7–102.7% and 88.9–105.5%, respectively), and matrix effect (99.5–111.0% and 98.9–115.1%, respectively) were defined and compared. Finally, the validated methods were applied to identify and quantify carbamazepine and its metabolite in hair in patients who received CBZ for medical reasons.

1. Introduction

Carbamazepine, which was synthesized in 1953 by W. Schindler, belongs to the group of versatile drugs [1]. Its main application is in the treatment of epileptic seizures, but it also has neurotrophic, antidepressant, and analgesic effects. CBZ is widely used in the treatment of epilepsy, often in combination with other anticonvulsants, such as valproic acid [2]. It is a highly lipophilic substance, it is quickly distributed into tissues (logP = 2.45). After administration of a single dose (200–1200 mg), carbamazepine in the body is slowly and incompletely absorbed from the gastrointestinal tract, reaching the maximum concentration in the blood after 2–24 h (the absorption time depends on the form of the drug administered; for the suspension it is a maximum of 2 h, and for tablets - 24 h). CBZ is metabolized in the liver mainly by CYP3A4 to e.g. its active and potentially toxic metabolite, carbamazepine-10,11-epoxide (CBZ-E) [1,2]. The serum concentration of the CBZ-E metabolite is generally 15–20% of the parent drug at therapeutic concentrations. There are reports showing that its % content increases with concomitant use of valproate and other anticonvulsants [2]. Some combined drugs, such as phenobarbital, phenytoin, and fluoxetine, can increase the blood level of CBZ. Therefore, the monitoring of CBZ and its metabolites in the blood is necessary to reduce the risks of adverse effects and controlling the results of therapy [3].

However, blood or serum analysis in therapeutic drug monitoring provides only real-time information. An alternative material such as hair is ideal for long-term control. Hair is a reliable and desirable biological matrix because it has a wide detection window, making it possible to provide information from months or even years [4]. In clinical toxicology, hair analysis can be used, among others, in the control of abstinence in addiction treatment programs, in drug concentration-monitored therapy, or screening of drug intake by mentally ill patients. Therefore, it is important to look for new reliable methods for determining substances from hair samples. The literature provides information on the determination of carbamazepine in the hair by

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various analytical techniques (e.g. HPLC-UV, LC-MS, GC-MS) and the dependence of carbamazepine level on the dose of the drug or the color of the patient's hair [5–10]. However, there are few reports in the literature on the content of CBZ-E in hair - one of the major potentially toxic metabolites of carbamazepine [6,11].

The main aim of this research was to develop a novel method using the capillary electrophoresis (CE) system coupled with a mass spectrometry detector (MS) for the determination of carbamazepine (CBZ) and its metabolite CBZ-E in human hair. The capillary electrophoresis technique coupled with mass spectrometry detection (CE-MS) can be an alternative technique to LC-MS separation [12]. Scientific reports indicate that the CE-MS technique is a useful tool for monitoring various psychoactive compounds (e.g. psychotropic drugs and biomarkers) in biological materials (e.g. urine, plasma, hair and saliva) [13–17]. To the best knowledge of the authors, there are no reports on the use of the CE-MS technique for the determination of CBZ and CBZ-E in human hair. The second goal of this research was to compare the CE-MS results with the liquid chromatography (LC) technique also coupled with the MS detector, using the RGB-12 algorithm (12 principles of White Analytical Chemistry (WAC)) [18,19].

2. Material and methods

2.1. Reagents, standards and materials

LC-MS grade reagents: acetonitrile, methanol, isopropyl alcohol, and analytical grade reagents: formic acid, acetic acid, ammonia solution, ammonium formate, ammonium acetate, and 20% water solution of highly sulfonated (α,β,γ)-cyclodextrins (HS(α,β,γ)CD) were purchased from Merck (Darmstadt, Germany). Ethyl acetate (HPLC grade) and an analytical grade 30% NaOH water solution were purchased from Avantor Performance Materials (Gliwice, Poland). Water (18.2 MΩcm, TOC < 5 ppm) was ultrapurified in a Milli-Q Plus system (Millipore, Bedford, MA, USA). Drug standards: carbamazepine (CBZ) and 10,11-epoxy-10,11-dihydro-carbamazepine (CBZ-E) were purchased from LGC Standards (Teddington, UK). The deuterated analogues of CBZ – carbamazepine-d10 (CBZ-d10) acquired from Lipomed AG (Arlesheim, Switzerland).

Drug stock solutions of CBZ and its metabolite (1 mg/mL) and the internal standard (IS, 0.1 mg/mL) were stored in methanol at − 20 °C. The stability of the drug stock solution was evaluated by a comparative analysis of solutions aged 2 and 4 weeks with freshly prepared ones, which revealed no significant differences in the analytical signal (t-Student test, confidence level of 95%). Spiking solutions were prepared daily by appropriately diluting stock solutions with water. Standard drug solutions, used in experiments to assess the extraction efficiency were prepared by diluting stock solutions with background electrolyte (BGE) or mobile phase (MP) to a concentration 4.4 ng/mg, in 45 mg of hair sample, while in the studies on matrix effects and recovery to concentrations: for CBZ: 1.1, 8.9 and 22.2 ng/mg (45 mg of hair sample) and for CBZ-E: 1.1, 4.4 and 8.9 ng/mg (45 mg of hair sample).

Control, drug-free hair samples (dark blond and brown) to develop the new methodology were received from healthy volunteers with no history of psychoactive drug intake. The samples were stored in sealed polyethylene bags at room temperature in a dry and dark place until analysis. Natural hair samples positive for CBZ were collected from person who had been treated with carbamazepine during routine autopsy. The post-mortem samples were originated from the Forensic Medicine Unit at the Department of Forensic Medicine of the Wroclaw Medical University and were classified as samples which that may contain such psychotropic drugs. (see Sections: *3.4. Case studies*).

2.2. Sample preparation and extraction conditions

Before analysis, all hair samples were carefully rinsed in the following sequence: methanol, water, and then methanol again to eliminate any external contamination. After drying at room temperature, each hair sample was cut into c.a. 1.5 mm long pieces, ground with a ball-grinder (Retsch, Haan, Germany), and 45 mg of powdered hair sample was weighed in an extraction vessel. In the optimization and validation steps, spiked hair samples were used.

A MARS 5 microwave-assisted sample preparation system (CEM, Matthews, NC, USA) equipped with 24 Xpress® PFA vessels (75 mL) was used for the isolation of the drugs from hair samples. 1 mL of 0.6 M NaOH and 3 mL of extraction solvent (ethyl acetate) were added to the 45 mg hair sample. The MAE process was carried out for 10 min at 60 °C. The temperature was ramped from room temperature in 5 min, using microwave power ranging from 480 to 800 W. After the MAE process, the content of each vessel was transferred to a plastic conical tube and centrifuged (10 min, 4000 rpm, 4 °C). In the next step, 3 mL form 4 mL of the organic layer was separated and evaporated to dryness under a stream of nitrogen at 40 °C. The dry residue was then dissolved in 500 μL of water, mixed (5 min, 2500 rpm, RT) and then centrifuged (10 min, 10,000 rpm, 4 °C). 100 μL of the extract was mixed with ammonium acetate to give a concentration of 1 mM ammonium acetate and analyzed by the CE-MS method. For LC-MS analysis, 100 μL of the extract was diluted with acetonitrile 1:1 (v/v) and analyzed. In the optimization step of the MAE extraction process, the internal standard (CBZ-d10) was added to the extracts after extraction and its concentration was 50 ng/mL.

2.3. CE-MS conditions

Separation of analytes was carried out in a PA 800 plus capillary electrophoresis system (Sciex, USA) coupled with a mass spectrometer MicrOTOF II (Bruker, Bremen, Germany) with electrospray ionization source (ESI) and time-of-flight analyzer (TOF). The separation of CBZ and its metabolite was performed for 30 min using the voltage of + 30 kV in a fused silica capillary (75 μm i.d., 100 cm length, Sciex, USA), using 10 mM ammonium acetate (pH = 6.8) as the background electrolyte (BGE), after filling the capillary with 1% HSβCD in 10 mM ammonium acetate (4.83 kPa for 30 s). The capillary temperature was set at 25 °C. The injection of the samples was hydrodynamic, applying a pressure of 4.83 kPa for 6 s. Before analyzes, the capillary was rinsed for 30 min with 1 M NaOH, while between measurements it was rinsed with water (2 min, 172.37 kPa), MeOH (2.5 min, 206.84 kPa) and then again with water (5 min, 137.90 kPa).

MS detection was performed in the positive ion mode with ESI conditions: nebulizer pressure: 0.4 bar, dry gas: 4.0 L/min heated to 180 °C and capillary voltage − 4500 V. The mass resolving power of the instrument was over 16 000 and profile spectra were acquired in the mass range 50–1000 *m/z*. Mass calibration was carried out using sodium formate clusters after each run according to the procedure given by Bruker. Data were collected using Compass DataAnalysis 3.2 software (Bruker). The results were recalculated on the basis of an extracted ion chromatogram for the expected ions of the analytes [M + H] +, calculated using IsotopicPattern software (Bruker): CBZ - 237.1022 *m/z*, CBZ-E - 235.0972, CBZ-d10 (IS) - 247.1650. The sheath liquid delivered into the ESI source with the flow rate of 180 μL/h, consisted of a mixture of isopropanol/H2O, 1:1, v/v with 0.2% HCOOH.

2.4. LC-MS conditions

LC analysis was performed using an UltiMate 3000 RS liquid chromatography system (ThermoScientific, USA) coupled with a mass spectrometer with electrospray ionization source (ESI) and a time-of-flight mass analyzer (MicrOTOF-Q II, Bruker, Bremen, Germany) [20]. The separation of CBZ and its metabolite was carried out in a Hypersil Gold Phenyl column (50 mm × 2.1 mm I.D., particles 1.9 μm, injection: 5 μL, Dionex) at 25 °C. A mixture of 0.1% formic acid in water (A) and acetonitrile (B) was used as the mobile phase, in a gradient program (ACN: 0 min–10%, 3 min–95%, 5 min–95%, 8 min–10%, 12 min–10%), at a flow rate of 0.4 mL/min.

MS detection was performed in positive-ion mode with ESI conditions: nebulizer pressure: 2.5 bar, dry gas: 5.5 L/min heated to 200 °C, and capillary voltage − 4200 V. The mass resolving power of the instrument was over 18 000 and profile spectra were acquired in the mass range 50–1000 *m/z*. Data collection and MS calibration were performed as for the CE-MS method.

2.5. Method validation

The validation procedure was carried out according to the standard practices for the validation of the method by the Scientific Working Group on Forensic Toxicology (SWGTOX) [21].

The linearity of the methods for hair samples was tested within the range of 1.1 – 22.2 ng/mg for CBZ (1.1, 2.2, 4.4, 11.1, 22.2, 26.7 ng/mg) and for CBZ-E – 1.1 – 8.9 ng/mg (1.1, 2.2, 3.3, 4.4, 8.9 ng/mg). Calibration curves were calculated using peak-area ratios (analyte/IS). Deuterated CBZ-d10 was used as IS and its concentration of it (added to hair samples before MAE extraction) was constant and equal to 1.1 ng/mg.

The limits of detection (LOD) and quantification (LOQ) were calculated as the ratios of three times and ten times the standard deviation of the analytical signal (measured at a concentration level of 1.1 ng/mg, for CBZ and CBZ-E) to the slope of the calibration graph, respectively [22].

Precision (CV), recovery (RV) and matrix effects (ME) were investigated using hair samples spiked with the studied analytes at three concentration levels: 1.1, 8.9 and 22.2 ng/mg for CBZ and 1.1, 4.4 and 8.9 ng/mg for CBZ-E – close to low, middle and high values of the calibration range, respectively. For the CV, RV and ME parameters, the acceptance criterion was set at 15.0%, 100 ± 15.0%, and 100 ± 15.0% (± 20% for the lowest concentration), correspondingly [23]. In order to evaluate the precision of the method, five samples containing analytes of a given concentration were analyzed within one day, and then the measurements were repeated within three other days. In such a way, the within-day and between-day precisions were evaluated (by the one-way ANOVA approach). Recovery of the extraction step was expressed by the ratio of the analytical signal obtained for an analyte that had been added to a drug-free hair sample (n = 5, 3 repetitions) and then extracted, to the analytical signal for the analyte, which had been added at the same concentration to an extract of the sample (taking into account the enrichment factor) [23,24]. The matrix effect was calculated as the ratio of the analytical signal measured for an analyte added to a blank sample after extraction (n = 5; three-fold repetition of each sample) to the analytical signal for the analyte (at the same concentration) in a standard drug solution [23].

3. Results and discussion

3.1. Optimization of the MAE process

In the study, the procedure for the isolation of CBZ and its metabolite CBZ-E from human hair was applied using the MAE extraction method, modifying the procedure developed in the previous works [22,25]. Taking into account the pKa of CBZ (13.9), an alkaline extraction medium (0.6 M NaOH; pH = 10) was used, and ethyl acetate was chosen as the extraction solvent. For other conditions, see Section: *2.2. Sample preparation and extraction conditions*. The influence of time (10, 20 min) and temperature (50, 60, 70 and 80 °C) on the MAE process was investigated using the one independent variable method. The extraction efficiency (E) was chosen as the evaluation criterion to find out the most efficient MAE procedure. The E relating to an analyte was calculated as the ratio of the analytical signal (relative peak area) obtained for the analyte in the sample to that obtained for the standard drug solution. The MAE process was carried out on hair samples (45 mg) spiked with analytes (4.4 ng/mg, each). Then, after the MAE process, the extracts were spiked with IS (1.1 ng/mg), and analyzed using the CE-MS method. Each experiment was repeated five times. Additionally, standard drug solutions (containing the same concentrations of analytes and IS) were analyzed.

The extraction efficiency results were compared with each other using the function $\mathbf{F_{MAE}}$: $\mathbf{F_{MAE}} = \mathbf{(k^{2}*E)/SD_E}$ [22,25]. Where, **k** is the number of analytes with an extraction efficiency value exceeding 80% (max. **k** = 2), **E** is the mean extraction efficiency calculated for both analytes and $\mathbf{SD_E}$ is the mean value of the standard deviation of the extraction efficiency evaluated for 2 analytes (each at 4.4 ng/mg in the hair sample).

Summarizing this stage of the research, it was noticed that the longer time and the higher temperature of the MAE process, the decrease in the extraction efficiency of CBZ and its metabolite CBZ-E was observed (for MAE at 80 °C for 20 min, the extraction efficiency of the analytes was ~ 75% and ~ 55, respectively). Furthermore, the value of the $\mathbf{F_{MAE}}$ function was the lowest for these conditions (around 8.0). Additionally, the use of high temperatures during the MAE process (70 and 80 °C) resulted in poor reproducibility of the results (for both extraction times), which is reflected in the low values of the $\mathbf{F_{MAE}}$ function obtained for these conditions ($\mathbf{F_{MAE}}$ in the range 8–19). The best extraction efficiency of CBZ and CBZ-E from hair (at the level of ~ 100% and ~ 85%, respectively) was achieved under MAE conditions: 10 min at 50 and 60 °C, with better reproducibility of the results for the higher temperature. Finally, as the best conditions for MAE extraction, the process at 60 °C for 10 min was selected, and for these MAE conditions the maximum value of the $\mathbf{F_{MAE}}$ function was achieved ($\mathbf{F_{MAE}} = 125$). In comparison, for the MAE for 10 min at 50 °C, the value of the $\mathbf{F_{MAE}}$ was only 49. The effectiveness of the extraction of tested analytes from human hair under optimal conditions using the MAE/CE-MS method ranged from 86.4% for CBZ-E to 110.3% for CBZ, with good repeatability (CV), less than 10.0% (n = 5). The value of extraction efficiency above 100% may indicate the existence of matrix-derived interferences that affect the signal from the analyte. During method validation, this phenomenon was compensated by using a matrix-matched calibration process.

The comparison of the values of the $\mathbf{F_{MAE}}$ function obtained for the best conditions of MAE extraction for CE-MS and LC-MS methods are presented in Supplement 1.

3.2. Optimization of the CE-MS method

The aim of the research was to find a background electrolyte (BGE) that is compatible with the applied MS detector and at the same time effective in the separation of CBZ and CBZ-E. The selected analytes have different molar masses, which allows for their identification by *m/z* ion masses; however, ionization at the same time in the ESI source may cause a decrease in sensitivity through ion suppression. An experiment was carried out using standard analyte solution (500 ng/mL) dissolved in BGE ten times diluted with water.

In order to efficiently separate CBZ and CBZ-E using the CE technique, the composition of BGE was studied. For this purpose, electrolytes based on volatile acids (HCOOH, CH_3COOH) and organic salts (ammonium formate and acetate) of various concentrations (5, 10, 15 mM) and pH (5–9, addition of ammonia) were investigated. Organic modifier (ACN, MeOH) were also tested in various proportions (10, 20, 30%). The use of all the background electrolytes did not allow separation of the tested analytes. The addition of the organic factor resulted in an extension of migration times and deterioration of the separation process. In addition, along with the increase in the pH of BGE, a reduction in the migration time of the tested ions was observed, however, it did not have a positive effect on the separation process. In the next step, the addition of highly sulfonated cyclodextrins (HSCD) to BGE for the separation of CBZ and CBZ-E was examined. HSCDs are compounds that have numerous anionic sulfonic groups in their structure, whose ionization is possible over a wide pH range, and under the influence of the applied voltage in the capillary (normal polarity), they migrate toward the positive electrode (capillary inlet) and opposite to

the EOF (with which the analytes in the capillary move to the capillary outlet). In order to reduce the influence of the HSCD macromolecular cyclodextrins on the ESI ion source - which can lead to disruption of the ionization process and a decrease in method sensitivity - a solution of HSCD in BGE was introduced into the capillary before each sample analysis, followed by a separation process using BGE without added HSCD. When a voltage is applied in the capillary, the negatively charged HSCD migrates opposite to the analytes while interacting with the analytes and increasing the selectivity of the separation. During the research, various types (α, β, γ) and concentrations (0.5%, 1.0% and 2.0%) of HSCD were tested for the separation process of the analyzed compounds. The best results were obtained using 1% HSβCD in 10 mM ammonium acetate. The use of the highest concentration of cyclodextrin (2%) had a negative effect on the capillary (it was breaking); while the smaller ones (0.5%) were insufficient to separate CBZ from CBZ-E.

The optimal operating conditions of the mass spectrometer optimized using single variable optimization are shown in Section 2.3 *CE-MS conditions*.

The electropherograms of the separation of CBZ and CBZ-E under optimal CE-MS conditions are presented in Fig. 1.

3.3. Validation of the MAE/CE-MS and MAE/LC-MS methods

The optimized new methodology MAE/CE-MS, and the reference MAE/LC-MS method for determined CBZ and CBZ-E for the investigation of hair samples were validated [20,21,23]. The validation parameters are presented in Tables 1 and 2.

All measurements were carried out using hair blank samples, i.e. free of studied compounds. This was confirmed by the analysis of extracts from 10 hair samples (from 5 different sources) without the addition of the studied drugs and IS. Each set of analyses of spiked samples was also supported by the analysis of a drug-free sample to define the specificity of the method. No sample components that cause additive interferences were identified. Identification of a particular analyte (selectivity) was confirmed by comparing the accurately measured ion mass (of the given analyte) with the calculated mass and by comparing the relative migration time (Δt_m, the ratio of the migration time of the analyte to the migration time of the IS, t_m/t_{mIS}, for the CE method) or the retention time (RT, for the LC method) of peaks of distinctive ions with those of the CBZ and CBZ-E standards, taking into account a 2% wide detection window. The selectivity study revealed that the measured ion mass for each analyte met the 3 ppm criterion provided by the MS detector manufacturer. The stability of migration times and retention times, particularly the width of detection windows (2%), was confirmed by the analysis of spiked hair samples (at 3 levels).

The linearity of both MAE/CE-MS and MAE/LC-MS methods in the tested concentration range is acceptable for all analytes - the coefficient of determination (R^2) was not less than 0.999. The gained LOD and LOQ values enable the determination of the tested compounds in hair at the levels declared in the literature (analysis of hair samples collected from patients who had been treated with CBZ) [5,6,11]. Additionally, when the two methods are compared, lower LOD and LOQ limits were obtained for the LC-MS technique, although these are not significant differences compared to the CE method.

Moreover, the MAE/CE-MS and MAE/LC-MS methods for every tested level are characterized by a within-day precision lower than 10.5% and 7.8%, respectively; and by a between-day precision better than 14.5% (CE-MS) and 11.1% (LC-MS). The value of 15% was slightly exceeded for the lowest concentration of CBZ-E (15.6%) for the LC method; however, this value is still within the acceptance range (20% for the lowest concentration). In conclusion, better precision was obtained with the chromatographic method. For all the evaluated concentration levels of CBZ and CBZ-E in spiked hair, the calculated RV lies within the range of acceptance (100 ± 15%) for both methods. However, CBZ-E was characterized by a lower recovery than CBZ, but this is confirmed by the results of the extraction efficiency of this metabolite from hair samples, which was also about 85%. The matrix effect for both methods is also within the acceptance range (only for the LC method for the lowest CBZ-E level this value was 15.1%). Thus, it can be concluded that the matrix components do not significantly affect the obtained analytical signals.

On the basis of the obtained results, it was ascertained that both developed MAE/CE-MS and MAE/LC-MS methods are reliable analytical tools for human hair samples analysis.

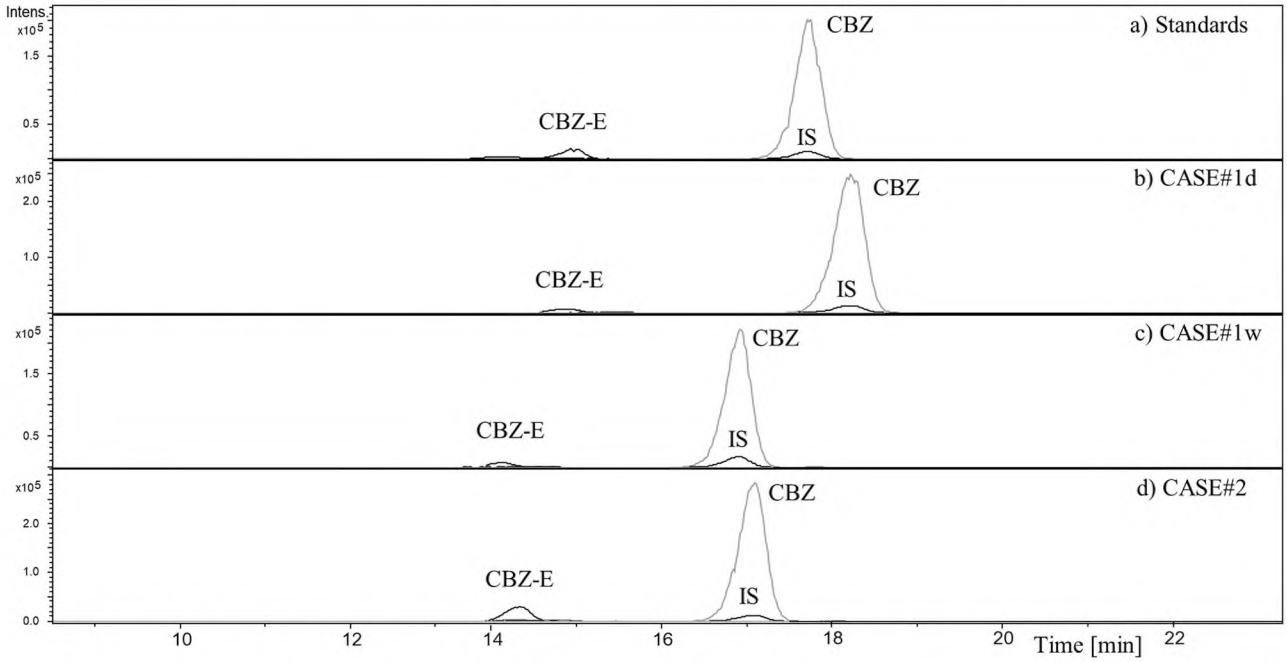

Fig. 1. Electropherograms of CE-MS separation of CBZ and CBZ-E: **a)** in standard solutions; **b)** and **c)** in hair extracts for CASE#1 (CASE#1d – dark hair, CASE#1w – white hair); and **d)** in hair extracts for CASE#2.
CE-MS method conditions: the voltage + 30 kV; the capillary temperature: 25 °C; BGE: 10 mM ammonium acetate after filling the capillary with 1% HSβCD.

Table 1
Comparison of validation parameters of the MAE/CE-MS and MAE/LC-MS methods for determination of CBZ and CBZ-E in hair – part I.

Method	Drug	Δt_m* (min) (CV %)	LOD [ng/mg]	LOQ [ng/mg]	Linearity range [ng/mg]	R^2
MAE/CE-MS	CBZ	0.99 (0.14)	0.36	0.86	1.1–26.9	0.9998
	CBZ-E	0.86 (2.0)	0.38	0.94	1.1–8.9	0.9993
Method	Drug	RT [min] (CV %)	LOD [ng/mg]	LOQ [ng/mg]	Linearity range [ng/mg]	R^2
MAE/LC-MS	CBZ	3.37 (0.23)	0.22	0.72	1.1–26.9	0.9994
	CBZ-E	3.14 (0.84)	0.17	0.56	1.1–8.9	0.9992

* the ratio of the migration time of the analyte and the migration time of the internal standard IS, t_m/t_{mIS}

3.4. Case studies

Hair samples were collected, for reason of check the reliability of new methodology from persons (*CASES #1 and #2*), taking CBZ for medical reasons. In each case, tests were performed on four samples and a blank hair sample: 45 mg each. An internal standard (CBZ-d10) was added to the samples (1.1 ng/mg) before MAE extraction. The extracts were then analyzed by both CE-MS and LC-MS methods, simultaneously. Natural hair samples from these persons (*CASES # 1 and #2*), were also used for the evaluation of the extraction process. A positive sample was extracted twice in sequence. What is so important, the second extraction revealed no trace of the analyte, confirming that a single extraction is sufficiently efficient.

The CASE#1 (a 44-year-old man with dark and white hair that had not been bleached or dyed) had taken 200 mg of Tegretol CR (CBZ) three times daily for 10 years. For this experiment, c.a. 1.5 cm of patient's hair from the whole head was collected. The dark hair (*CASE#1d*) and white hair (*CASE#1w*) was separated, then pre-treated and analyzed. In both cases, carbamazepine and its main metabolite (CBZ-E) were found in the hair, and the results of these tests are presented in Table 3 and in Fig. 1 and Fig. 2. The second hair sample (*CASE#2*) was collected from a 23-year-old male with dark hair (not bleached or dyed), suffering from epilepsy. The patient was treated with Neurotop retard (CBZ, slow release tablets), which had been taking 750 mg twice a day since he was a child. He was also taking Orfiril retard (sodium valproate) 600 mg twice daily. In this *CASE#2*, CBZ and CBZ-E were also detected in the hair samples and the results of this analysis using both analytical methods are presented in Table 3 and in Fig. 1 and Fig. 2.

In all three cases, the concentration of CBZ exceeded the concentration of its metabolite in the hair. This can be explained by a higher lipophilicity of carbamazepine compared to its metabolite (logP for CBZ-E = 1.97) [7] and its greater ease of penetration into the hair, thus there was a simpler incorporation of CBZ into the hair structure from the blood stream. Another reason is related to CBZ metabolism in the human body and the resulting serum concentrations of both compounds (the CBZ-E concentration in serum is generally 15–20% of the parent drug in therapeutic concentration [1]). Analysis of the obtained results shows that the CBZ concentration in white hair (*CASE#1w*) was much lower than in dark hair (*CASE#1d*) of the same patient, which was also observed in other research [5–7] (see: Table 3). Some studies confirm that the presence of hair pigment (melanin) facilitates the incorporation of drugs (especially alkaline [4,26]) into the hair structure. On the other hand, Mieczkowski et al. in their work [5] proved that the concentration of CBZ in hair is mainly influenced by the daily dose of the drug - the color of hair is significant, but it affects it to a lesser extent. Furthermore, the CBZ concentration in *CASE#2* (despite the higher daily dose) was only slightly higher than in *CASE#1d*. This may indicate that the concentration of CBZ in the hair is also influenced by other factors, i.e. individual variability, age, hair structure, and daily hair care, but also the type of used drug (e.g. slow release tablets result in lower peak plasma concentrations of CBZ than ordinary tablets).

Although there is a lot of information in the literature on the concentration of CBZ in hair, there are only few reports on the determination of CBZ-E in hair samples. In this study, it was observed that the concentration of CBZ-E practically did not differ between the *CASE#1d* and *CASE#1w* samples. However, in the *CASE#2* sample, four times more CBZ-E was detected than in the other two cases, which can be correlated with the daily dose of the drug, which in this case was 2.5 times higher. Potter et al. [2] came to interesting conclusions - in his work, he points to the influence of other drugs on the levels of CBZ-E concentration in the serum, which can translate to some extent into its concentration in the hair. It has been observed that in patients taking drugs containing CBZ and valproate simultaneously, an increase in serum CBZ-E concentration is observed compared to patients taking CBZ only. This fact may also explain the differences in CBZ-E concentrations in *CASE#1* and *CASE#2*.

Comparison of the results of the determination of CBZ and CBZ-E in hair using the MAE/CE-MS and MAE/LC-MS methods shows that the obtained results were convergent and that any discrepancies were slight and fell within the error limits.

Table 2
Comparison of validation parameters of the MAE/CE-MS and MAE/LC-MS methods for determination of CBZ and CBZ-E in hair – part II.

Method	Drug	Expected concentration [ng/mg]	Precision, CV [%]		Recovery[a] RV [%] (n = 5) (CV%)	Matrix effect[a] ME [%] (n = 5) (CV%)
			Within day (n = 20)	Between day (n = 20)		
MAE/CE-MS	CBZ	1.1	7.96	10.6	102.7	105.3
		8.9	6.24	7.26	101.8	111.0
		22.2	2.11	6.91	96.8	99.5
	CBZ-E	1.1	10.5	13.4	88.4	110.0
		4.4	7.39	14.5	87.7	102.6
		8.9	6.37	8.51	88.3	106.4
MAE/LC-MS	CBZ	1.1	7.80	11.1	100.8	103.1
		8.9	3.52	10.3	102.9	98.9
		22.2	0.26	2.16	105.5	100.9
	CBZ-E	1.1	6.97	15.6	89.9	115.1
		4.4	2.23	8.35	88.4	111.1
		8.9	1.18	6.12	90.3	110.9

[a] Number of repetitions in one day (5 analyses).

Table 3
The concentration of CBZ and CBZ-E in human hair (n = 4) obtained using the MAE/CE-MS and MAE/LC-MS methods and compared with the concentration reported in the literature [5,6,8,10,11].

Method	CASE NO	Color of hair	Dose/day [mg/day]	Concentration of CBZ [ng/mg]	Concentration of CBZ-E [ng/mg]
MAE/CE-MS	CASE#1d	dark	600	21.64 ± 1.16	2.28 ± 0.23
	CASE#1w	white	600	15.21 ± 1.14	2.16 ± 0.10
	CASE#2	dark	1500	23.99 ± 1.32	9.91 *** ± 0.24
MAE/LC-MS	CASE#1d	dark	600	20.12 ± 1.10	1.98 ± 0.11
	CASE#1w	white	600	13.78 ± 1.04	1.87 ± 0.08
	CASE#2	dark	1500	23.49 ± 1.66	8.99*** ± 0.96
HPLC[5]		-*	525**	18.20**	- *
HPLC[10]		-*	-*	0.6 – 63.7	- *
HPLC-UV[11]		-*	400	10.90	- *
GC-MS[6]	P#1	dark	-*	22.0	0.32
		white	-*	18.0	0.22
	P#2	dark	-*	15.0	0.40
		white	-*	14.0	0.27
	P#3	dark	-*	6.0	0.17
		white	-*	6.0	0.17
GC-MS[8]		-*	200–400	2.8 – 22.5	-*

*no data in literature **mean concentration (40 patients) *** this value is approximate, as it exceeds the upper range of the calibration curve (8.9 ng/mg)

3.5. Comparison of MAE/CE-MS and MAE/LC-MS methods

In order to compare and evaluate the effectiveness of the MAE/CE-MS and MAE/LC-MS methods, they were analyzed using the RGB-12 algorithm according to the methodology of the 12 principles of White Analytical Chemistry (WAC) (see Fig. 3). WAC [19] takes into account key criteria affecting the quality of the method: analytical (red), green (green), and practical (blue). In reference to the RGB color model, according to which mixing of red, green and blue light beams gives the impression of whiteness, a white analytical method shows the coherence and synergy of the analytical, ecological and practical attributes. Whiteness can also be quantified, based on the assessment of individual principles, as a convenient parameter useful in comparisons and selecting optimal method.

According to this model, the MAE/CE-MS method is more environmentally friendly (green) than the one using liquid chromatography. This result is definitely influenced by the type of reagents used, as well as their volume and toxicity. In the case of capillary electrophoresis, we use less hazardous reagents, and the total volume required for the preparation and analysis of one sample is also smaller than that required in the LC technique. On the other hand, the analysis of a sample using the capillary electrophoresis technique with MS detection is more than twice as long as the LC technique, and the operation of the CE system is more complicated. Hence, it follows that the MAE/LC-MS method is bluer. Analyzing the analytical aspect, the MAE/LC-MS method achieves lower detection and quantification limits and is characterized by a better repeatability of results than CE technique, hence the MAE/LC-MS method is redder. In summary, considering the whiteness of the method (100%), which is the result of the defined significance of the primary colors, the MAE/LC-MS method obtained a better result (85.6%), while the MAE/CE-MS method: 83.1%, but the difference between methods is accepted.

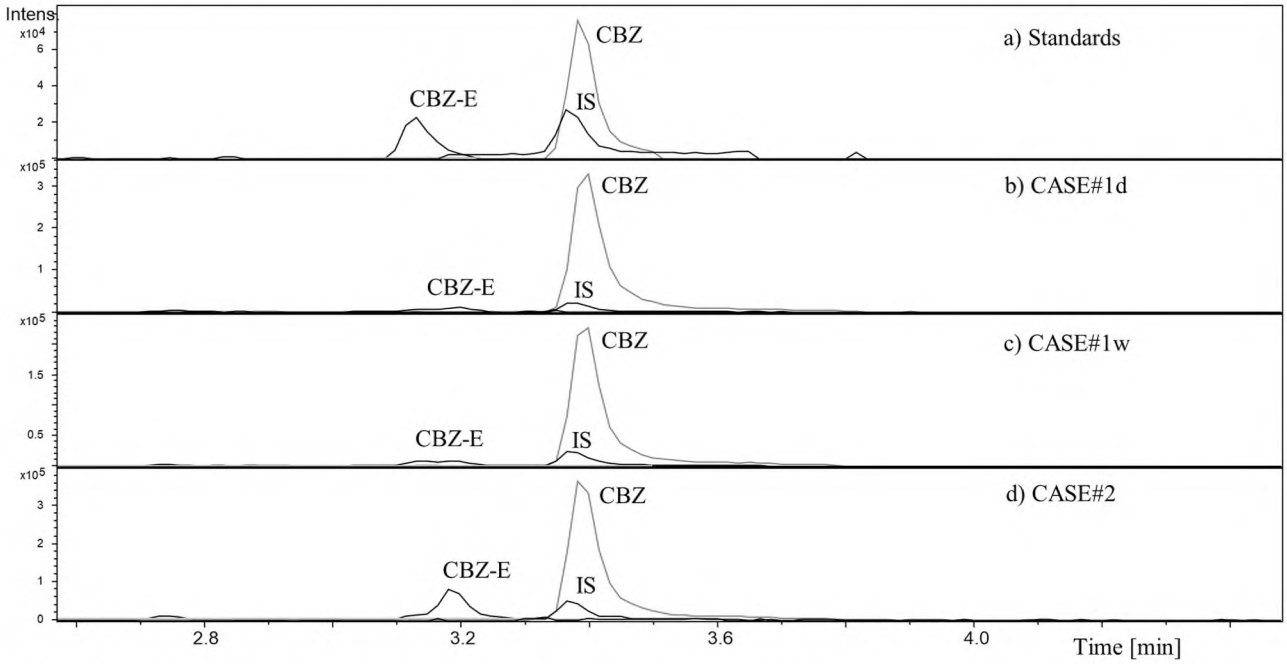

Fig. 2. Chromatograms of LC-MS separation of CBZ and CBZ-E: a) in standard solutions; b) and c) in hair extracts for CASE#1 (CASE#1d – dark hair, CASE#1w – white hair); and d) in hair extracts for CASE#2.
LC-MS method conditions: the mobile phase: 0.1% formic acid in water (A) and acetonitrile (B) in a gradient program: ACN: 0 min–10%, 3 min–95%, 5 min–95%, 8 min–10%, 12 min–10%); the flow rate of 0.4 mL/min; the temperature: 25 °C.

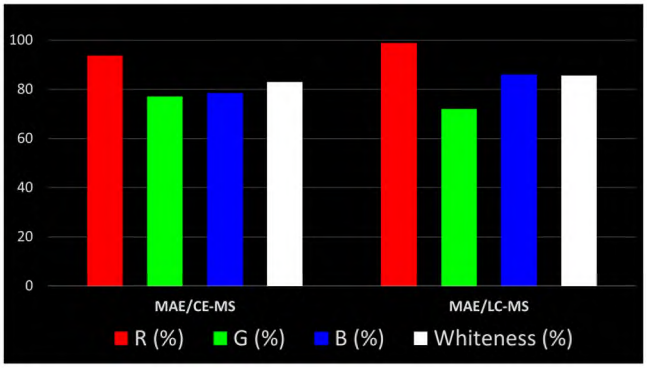

Fig. 3. The effectiveness of the MAE/CE-MS and MAE/LC-MS methods, compared and evaluated using the RGB-12 algorithm according to the methodology of the 12 principles of White Analytical Chemistry (WAC). WAC [19] takes into account key criteria affecting the quality of the method: analytical (red; **R**), green (green; **G**), and practical (blue; **B**). The obtained whiteness (%) value for the MAE/LC-MS method is 85.6%, while for the MAE/CE-MS method is 83.1%.

4. Conclusions

The aim of this study was to determine CBZ and its metabolite CBZ-E in human hair samples using the CE technique coupled with MS detection. During the research, it was possible to find an optimal separation buffer that allowed effective separation of the tested compounds and that was also compatible with the detector used. The appropriate extraction conditions of MAE allowed for the effective isolation of the tested analytes from human hair samples. The analytical and validation parameters of the MAE/CE-MS method, compared to those of the MAE/LC-MS method, are satisfactory and acceptable; and they confirm that the developed method can be a reliable analytical tool in the analysis of hair samples for clinical and forensic purpose.

CRediT authorship contribution statement

Aneta Woźniakiewicz: Methodology, Investigation, Visualization, Validation, Writing - original draft. **Renata Wietecha-Posłuszny:** Visualization, Conceptualization, Project administration, Supervision, Writing - review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.jpbao.2023.100009.

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