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DETERMINATION OF CARBAMAZEPINE IN HAIR SAMPLES BY HIGH PERFORMANCE LIQUID CHROMATOGRAPHY WITH ULTRA-VIOLET DETECTION

ODREĐIVANJE KARBAMAZEPINA U UZORCIMA KOSE METODOM VISOKO EFIKASNE TEČNE HROMATOGRAFIJE SA ULTRALJUBIČASTOM DETEKCIJOM

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Apstrakt

Analiza uzoraka kose nalazi primenu u potvrdi prethodnog uzimanja leka. Ove informacije mogu biti od koristi lekaru u smislu evaluacije istorije uzimanja leka i u različitim sudsko-medicinskim slučajevima. U radu je opisana metoda visoko efikasne tečne hromatografije sa ultraljubičastom detekcijom (HPLC-UV) za određivanje karbamazepina u uzorcima kose. Metoda je primenjena na uzorcima kose 26 bolesnika na terapiji ovim lekom. Razdvajanje leka od komponenti matriksa postignuto je reverzno-faznom hromatografijom na C18 koloni, sa mobilnom fazom metanol-voda-sirćetna kiselina (65:34:1) protoka 1,0 mL/min. Retenciono vreme karbamazepina bilo je 3,95 min. Nakon pranja sa dihlormetanom i inkubacije u rastvoru natrijum hidroksida, uzorci su pripremani alkalnom ekstrakcijom (pH 10) sa hloroformom. Kalibraciona kriva bila je linearna u opegu od 2,5-37,5 ng/mg kose. Srednji prinos ekstrakcije iznosio je 88,38 ± 8,33 %. Limit detekcije (LOD) i limit kvantifikacije (LOQ) bili su 1,2 ng/mg and 1,7 ng/mg kose. Koeficijent varijacije metode iznosio je 2,7%. Opisana metoda je brza, precizna, tačna i jednostavna i primenjena je u određivanju koncentracija karbamazepina u uzorcima kose bolesnika na terapiji ovim lekom. Uzorci kose mogu se primeniti kao potvrda i za dobijanje podataka o istoriji uzimanja ovog antiepileptika.

Ključne reči/ Key words
carbamazepine, hair, HPLC, determination/karbamazepin, kosa, HPLC, određivanje

INTRODUCTION

A development of analytical methods makes possible to use hairs in toxicological analysis for documentation history of drug administration. Serum (and also urine) analysis is effective for determining drug use only within the few days prior to sample collection. Long-term drug use may go undetected if the individual refrains from drug use for a few days before the sample collection. Hair analysis is a promising alternative to serum and urine analysis because it can provide a long term record of drug use. This has importance in therapy applying of antiepileptics.

Carbamazepine is the most applying antiepileptic drug, which has been used for more than three decades as the drug of first choice for the treatment of trigeminal neuralgia and for both generalized and partial seizures (1).

After oral administration it is absorbed slowly, but almost completely and distribute in blood and all tissues. It incorporates into the growing hair shaft from blood that supplies the hair follicle. (2)

Literature data shows that hair concentration over time has close relation to the plasma concentration. (3-11)

The most applying methods for determination of carbamazepine in biological materials use high performance liquid chromatography with ultraviolet or photodiode detection (HPLC-UV, HPLC-PDA) (7,9,11-16) and immunoassay (FPIA) (1,17-19). In literature has been described gas chromatography with mass spectrometry (20-21) and liquid chromatography with mass spectrometry method (22-23).

We described a simple, accurate and precise HPLC-UV method for determination of carbamazepine in hair samples.

METHODS

Materials

Analytical standard of carbamazepine (100 % s.s.), was obtained by Sigma.

Methanol, glacial acetic, sodium hydroxide, acid and chloroform were of HPLC and p.a. purity, obtained from MERCK. Ammonium hydroxide 25% was p.a. purity obtained from J. T. Baker. Water was purified by Millipore Milli-Q system.

Chromatography

The method used high performance liquid chromatograph LKB 2150 binary pump with Waters 2487 dual λ Absorbance Detector and Clarity Lite Software.

The mobile phase was mixture of metanol-demineralized water-glacial acetic acid (65:34:1 v/v/v), which has filtrated and degassed by membrane degasser.

Flow rate of mobile phase was 1 mL/min. through column Lichrospher 100 RP-8 (5 µm) 250-4, (Merck) with guard column Lichrochart 4 - 4 RP-8, at the room temperature, with injector loop volume of 175 µL. Detection of carbamazepine was performed on 285 nm. Retention time of carbamazepine was 3.95 minutes.

Sample preparation

Stock standard solution of carbamazepine was prepared by dissolving 10 mg in 10 ml methanol and stored at -4ºC. Other concentrations of carbamazepine were made by diluting stock standard solutions with mobile phase to achieve calibration concentrations expected to meet in hair of treated patients.

The patients who has ben received carbamazepine for at least six month were include in this study. Hair samples were cut from the head area, as close as possible to the skin of the posterior vertex in a quantity of 200 mg. Hairs of healthy individuals were used as a blank control.

Hair samples were washing two times with 5 mL of dichlormethane. After washing, samples were dried between filter paper on room temperature, in stream of air. Dry hair samples were cut into segments and pulverized. In 20 mg of pulverized hair was added 1 mL of 1M NaOH and samples were incubated 14 h on 45°C. After incubation and cooling on room temperature pH was adjust on 10 with 1M HCl. Carbamazepine was extracted on mechanical shaker for 20 minutes and centrifuged on 3000 rpm for 10 min. After centrifugation organic layer was separated and evaporated in stream of air. Dry extracts were reconstituted in mobile phase and analyzed by HPLC-UV method on 285 nm.

Calibration and quality control samples were prepared by adding carbamazepine solution in blank (”drug-free”) human hair. The amounts of carbamzepine in spiked hair ranging from 2.5 to 37.5 ng/mg. Concentrations of quality control samples were 2.5, 10 and 37.5 ng/mg.

Table 1. Calibration curve for carbamazepine in hair

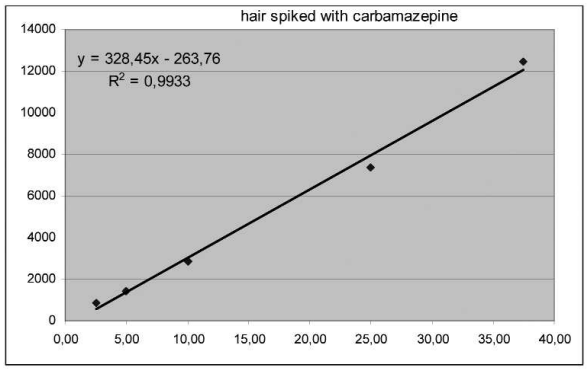
conc. (ng/mg)	AUC for spiked hair
2.5	860.00
5.0	1416.68
10.0	2859.98
25.0	7354.15
37.5	12466.01

Results

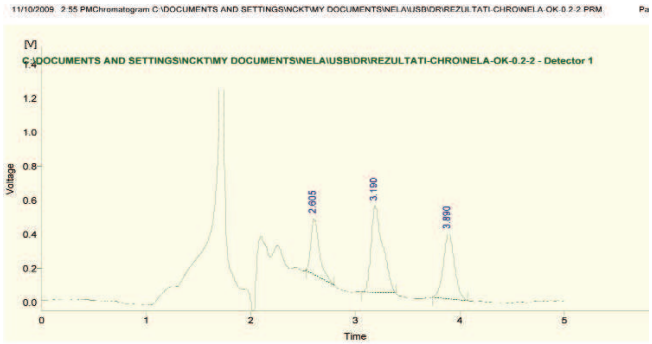
Carbamazepine concentrations were determined using weighed linear regression function. In Table 1 has shown calibration curve for carbamazepine in hair.

The correlation coefficient for hair spiked by carbamazepine was 0.9933. Picture 1 shows calibration curve for spiked serum and saliva.

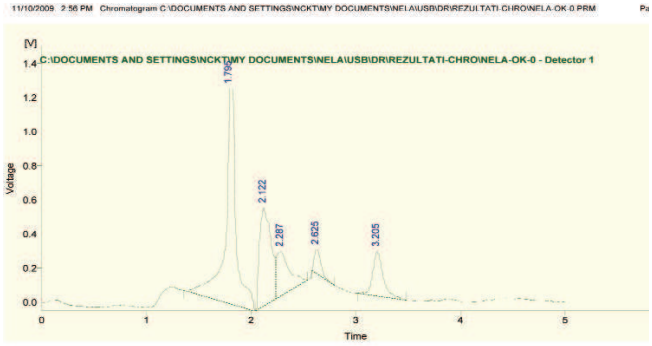
Recovery was determined, for each concentration, as the mean of three samples by comparing the peak areas of the extracted and non-extracted samples.



Picture 1. Calibration curve for spiked hair



Picture 2a. Chromatogram of blank hair



Picture 2b. Chromatogram of spiked hair

Retention time for carbamazepine in hair samples was 3,95 ± 0,022 min.

Picture 2 shows chromatograms of drug free and hair spiked by carbamazepine solution concentration of 10 ng/mg.

The precision of the method was assessed by calculating coefficient of variation (CV) for measured parameter of the method (peak area of carbamazepine) and determined on the same day (n=7). The inter- and intra-day coefficients of variation (CV) for spiked serum and saliva has shown in Table 2

The spiked hair extracts were stable when kept in the refrigerator for 48 hours. Mean 96.21 % (93.82 - 104.06).

Analytical recovery for hair spiked by carbamazepine was 88.38 ± 8.33 % (from 81.22 to 97.51 %). In Table 4. has shown limit of detection (LOD), limit of quantitation (LOQ), standard deviation (SD) and coefficient of correlation (CV) for spiked hair.

Unknown concentrations of carbamzepine in hair samples of patients were calculated using the corresponding factor from calibration curve. The factor is calculated from the mean value peak-area of carbamazepine for each mentioned concentration. Calibration curve has gotten after analysis of hair spiked by carbamazepine standard solution ranged from 2.5 to 37.5 ng/mg. Linear regression was y= 16422x - 263,76.

conc. (mg/L)	Intra day CV (%)	Intra day CV (%)
0.05	0.64	4.00
0.20	0.58	6.34
0.75	3.16	3.29

Table 2. Inter-day and intra-day coefficients of variation (CV) for spiked hair

LOD (ng/mg)	LOQ (ng/mg)	SD	CV (%)
1.2	1.7	23.21	2.70

Table 3. Limits of detection (LOD), limits of quantitation (LOQ), standard deviations (SD) and coefficients of variation (CV), for spiked hair

patient	hair (ng/mg)	serum (µg/mL)	patient	hair (ng/mg)	serum (µg/mL)
1	14.83	4.71	14	15.03	5.73
2	5,80	1.80	15	10.45	7.52
3	10.60	2.10	16	13.48	3.87
4	19.25	5.20	17	15.49	5.84
5	13.79	4.98	18	9.91	2.99
6	7.82	2.89	19	6.24	5.45
7	19.30	6.89	20	9.13	2.85
8	12.98	5.65	21	18.88	6.01
9	16.37	4.58	22	5.78	1.51
10	19.92	6.92	23	5.03	2.47
11	10.59	4.50	24	9.61	2.42
12	14.92	4.43	25	6.69	2.78
13	6.01	1.77	26	15.98	3.57

Table 4. has shown hair and serum carbamazepine concentrations of 26 patients on antiepileptic therapy.

Hair constituents did not interfere in carbamazepin assay.

The method was applied to determine the concentration of carbamazepine in patient's hair or samples. We have calculated correlation of carbamazepine concetrations in hair with determined concentration in serum of the same patients. HPLC-UV method for the determination of carbamazepine serum concentration after alkaline liquid-liquid extraction with chlorofom has been described by Djordjevic et al.

Discussion

It is known from clinical experience that some patients on antiepileptic therapy with carbamazepine try to alter their medication, reduce or even stop it, which sometimes may lead to unsatisfactory seizure control. The possibility of determining the hair levels of carbamazepine will therefore indicate whether or not a proper chronic use has been followed, and not only the reflection of day to day use that a blood sample provides. Hair acts as a tape recorder that continuously stores all data about the carbamazepine use history of the subject.

In process of hair analysis the most important is destroying of hair. Applyed condition have to destroy protein structure of hair, but not to change chemical structure of drug which have been determined. There are different methods for sample preparation to determinate carbamazepine level.

Tsatsakis et al. prepared hair samples by adding of 2 M NaOH solution, and heated it for 15 min at 80°C. After that the hair was dissolute by adding of concentrated HCl solution for pH adjusting on 1. It was heated for 20 min at 80°C and cooled at room temperature. Carbamazepine was extracted by alkaline (pH 9) solid-phase extraction on C18 cartridges with dichloromethane (1).

Wiliams et al. digested hair in 1.5 M NaOH overnight at 40°C. After cooling to room temperature the sample was neutralised with 1.5 M HCl and buffered by the addition of 0.1 M phosphate buffer (pH 7.6). They performed liquid-liquid extraction with methyl-tert-butyl ether (9).

We also performed alkaline dissolution. Hair was digested, after washing in dichloromethane, with 1 M NaOH at 40°C for 14 h. After cooling at room temperature pH was adjusted to 10 with 1M HCl. Carbamazepine was extracted from dissoluted hair samples by liquid-liquid method with chloroform at pH 10. Our result shows very good recoveryes (88.38 ± 8.33 %).

We had used mobile phase contained methanol-water-glacial acetic acid (65:34:1) described by Djordjevic et al. (16). Applying of this mobile phase we had reached good sensitivity. Compounds of matrix did not interfere with analyte (Picture 2a and 2b).

Reverse Phase HPLC analysis of the hair extracts was performed on C18 column using a mobile phase of acetonitrile/methanol/water (9:37:54) at a flow rate of 1.5 ml/min. The column eluate was monitored at 214 nm (9).

We also used C18 column for separation of carbamazepine from matrix, but UV detection was performed on 285 nm, which is wavelength where carbamazepine has maximum of absorbance. On that wavelength sensitivity of the method is the best. Moreover, compounds of matrix could be interfering with carbamazepine on lower wavelength such us 220 or 230 nm.

After analysing of samples we calculated carbamazepine concentration using the corresponding factor from calibration curve.

Mean carbamazepine concentration in hair after immunoassay was 28 ng/mg, and after GC analysis 26,6 ng/mg. Mean blood concentration was 6,2 mg/L (11)

Our results have shown lower carbamazepine concentration in hair. That is in compliance with patients lower concentration in serum. Mean carbamazepine concentration in hair and serum were 11.84 ng/mg and 4.29 µg/mL respectively.

Concentrations in hair samples of 30 patients on carbamazepine therapy longer than 6 month were 1,2 to 57,4 ng/mg. They correlate well with daily doses of carbamazepine. Coefficient of correlation was 0,793 ($p < 0.0001$) (3)

Segmental analysis of hair samples has shown that there was good correlation between history and time from beginning of therapy and distribution through the hair length. Analysis was performed on patients hair samples which were on therapy for 16 month (4).

Williams et al. showed that there were relatively small intrapatient variation in hair concentration over time and its close relation to the plasma concentration suggested that hair analysis may be a complementary and useful technique in monitoring drug-taking behavior (5).

Results of Mieczkowski have shown that coloring of hair had no influence on carbamazepine concentration. The

analysis reveals that there is a significant relationship between dose and concentration in hair (6-7).

Psiliakis et al. have shown that there werenžt significant diference between carbamazepine concentration after analizing by FPIA i HPLC. The concentrations depended on dose. There was a significant linear regression correlation of the carbamazepine concentrations in hair and serum samples of patients who has been treated long-term with this drug (8).

There was strong correlation between carbamazepine concentrations in plasma and hair (9).

We also proved that carbamazepine concentrations correlate well ($r= 0.7829$, $p< 0.001$).

Hair analysis also has been useful for diferentiation of chronic from acute carbamazepine intoxication. (10)

Conclusion

Hair testing is a subject of growing interest not only in the field of forensic science but also in clinical pharmacology. The HPLC-UV method described here is rapid, sensitive and simple for determination of carbamazepine in hair samples. The findings of this study demonstrate that monitoring of hair carbamazepine concentrations, the most used antiepileptic drug, could be useful technique for confirmation and history of therapeutic applying of this antiepileptic drug.

Abstract

A typical use of hair analysis is the documentation of previous drug administration. Those information would be valuable to physicians in order to evaluate past medical history and also for various medico-legal purposes. We had described high performance liquid chromatography method with ultraviolet detection (HPLC/UV) for determination of carbamazepine in hair samples. The method was included hair samples from 26 patients on carbamazepine therapy. Separation of the drug from matrix is achieved by reversed-phase chromatography on a C18 column, with a mobile phase of methanol-water-acetic acid (65:34:1) at a flow-rate of 1.0 ml/min. Retention time of carbamazepine was 3.95 min. Samples were prepared by alkaline extraction (pH 10) by chlorophorm after washing samples in dichlormethane and incubating with sodium hydroxide. Calibration curves were linear in the range of 2.5-37.5 ng/mg. Mean recoverie of spiked hair was 88.38 ± 8.33 %. Limit of detection (LOD) and limit of quantification (LOQ) were 1.2 ng/mg and 1.7 ng/mg respectively. The method precision was carried out with coefficient of variation of 2.71 %. Described method is rapid, precise, accurate and simple, and applied for quantitative determination of carbamazepine in patients' hair of after therapy applying. Hair samples could be used for confirmation and history of therapeutic applying of this antiepileptic drug.

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