Determination of Phenytoin and its Major Metabolite in Human Serum by MEKC

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Abstract

Determination of phenytoin in biological fluids is necessary due to its non-linear pharmacokinetics and narrow therapeutic index. A simple and rapid micellar electrokinetic chromatographic method was developed for simultaneous determination of phenytoin and its main metabolite, 5-(4-hydroxyphenyl)-5-phenylhydantoin, in human serum. The separation was carried out using fused-silica capillary and UV detector at 214 nm. The background electrolyte consisted of borate buffer (10 mM, pH 10.5) with 50 mM sodium dodecyl sulphate as pseudostationary phase. The calibration graphs were linear at the concentration range of 5–30 mg/mL, and the detection limits for phenytoin and 5-(4-hydroxyphenyl)-5-phenylhydantoin added to blank serum were 1.8 and 1.5 mg/mL, respectively. The obtained results demonstrated that the proposed method is suitable for the serum phenytoin analysis in terms of selectivity and simplicity.

Introduction

Phenytoin (PHT) is one of the first generation antiepileptic drugs, which is widely used in the management of generalized and partial seizures. Non-linear pharmacokinetic and narrow therapeutic range of PHT are well-proven. It is mainly metabolized in the liver to form 5-(4-hydroxyphenyl)-5-phenylhydantoin (4HPPH) via a saturable pathway at PHT plasma concentrations of more than 10 mg/L. PHT has also a narrow therapeutic range (10–20 µg/mL), interacts with a wide variety of drugs, and has several adverse effects. Therefore, therapeutic drug monitoring of PHT and determination of its main metabolite levels are essential to get a suitable efficacy with the minimum adverse effects (1).

A number of analytical methods have been developed to determine PHT and 4HPPH levels in biological fluids. Conard et al. used a previously described gas chromatographic method to measure PHT and 4HPPH levels in human serum, saliva, and hyperplasic gingival samples (2). Two other groups applied separate high-performance liquid chromatographic (HPLC) methods for simultaneous measurement of PHT and 4HPPH in serum (3,4). Although their methods were sensitive, the procedures used for sample preparation were complicated. Kabra and Marton developed a HPLC method to determine 4HPPH levels in human urine and did not apply it to plasma (5). Overall, these methods provide very sensitive techniques for drug assessment; however, they are costly methods. HPLC methods are also harmful to the environment because of using large amounts of organic solvents. Immunological assays provide rapid and convenient methods in clinical laboratories, but they cannot be used for simultaneous determination of PHT and 4HPPH because the antibody used for PHT measurement cross-reacts with its metabolite, which leads to an overestimation of PHT concentration (6). Capillary electrophoresis (CE) can overcome these problems because it offers very selective, cost-effective, and ecologically safe methods. A number of CE methods have been described for simultaneous determination of PHT and other antiepileptic drugs in serum (7–13), but there is no report on simultaneous analysis of PHT and 4HPPH with this method. Therefore, considering the advantages of CE technique over other analytical methods, development of a CE analytical method for this analysis would be of great value. Micellar electrokinetic chromatographic (MEKC) method is a kind of CE method that uses a pseudo-stationary phase to improve the selectivity of analysis and provide the possibility of analysis of non-ionized analytes. The aim of the present study is to develop a MEKC method for simultaneous determination of PHT and its major metabolite, 4HPPH, in human serum.

Experimental

Chemicals

Boric acid, sodium dodecyl sulphate (SDS), and 5-(4-hydroxyphenyl)-5-phenylhydantoin (4HPPH) were purchased from Sigma (St. Louis, MO). Sodium hydrogen phosphate, sodium tetraborate, TRIS, and extra-pure ethyl acetate were purchased

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from Merck (Darmstadt, Germany). Sodium hydrogen carbonate was from Panreac (Barcelona, Spain). Phenytoin (PHT) was a gift from Alhavi Pharmaceutical Company (Tehran, Iran), and sodium diclofenac (internal standard) was kindly provided by Sobhan Pharmaceutical Company (Rasht, Iran).

Preparation of standards and solutions

The stock solutions (1 mg/mL) of PHT and 4HPPH were prepared in methanol and stored at –20°C until use. A 1 mg/mL stock solution of sodium diclofenac, which was used as internal standard (IS), was made in distilled water and stored at 4°C. Standard solutions were prepared daily by diluting stock solutions with 10 times diluted background electrolyte (BGE). The BGE was prepared using a suitable buffer containing various amounts of SDS and filtered through a membrane filter (GHP, 0.45 µm, 47 mm, Pall Corporation).

Sample pre-treatment

Liquid phase extraction was used to prepare serum samples. A 50 µL of blank serum sample was added to 50 µL of standard solution containing suitable amounts of PHT, 4HPPH, and IS prepared in distilled water. After vortex-mixing for 30 s and adding 200 µL of saturated sodium hydrogen carbonate and 1.0 mL of ethyl acetate to the solution, the mixture was vortexed for another 2 min followed by centrifugation at 1000 g for 5 min. An 800-µL volume of the organic layer was transferred to a 1.5-mL microtube and evaporated under gentle stream of nitrogen. The residue was re-dissolved again in 80 µL of 10 times diluted BGE and injected into the capillary.

Apparatus and operating conditions

All analyses were performed using a Prince capillary electrophoresis (Prince Technologies B.V., Emmen, the Netherlands) equipped with a Jasco CE-971 UV/Vis detector (Jasco Corporation, Tokyo, Japan) operating at 214 nm. Detector's digital outputs were collected using Teifgostar software (Teifgostar Co., Tehran, Iran) for further numerical analyses. The separation column was an uncoated fused-silica capillary (50 µm i.d., 375 µm o.d., 90 cm total length, 72 cm effective length). Sample injections were carried out by pressure mode at 100 mbar for 0.30 min. The separation voltage of 30 KV was applied, and the temperature was maintained at 25°C. Before each run, the capillary was rinsed with 0.1 M NaOH, water, and running buffer each for 3.5 min. The new capillary was conditioned with 1 M NaOH, 0.1 M NaOH, and water before use.

Results and Discussion

Optimization of the background electrolyte

In order to achieve a suitable MEKC separation, type, pH, and concentration of the buffer and also the concentration of the surfactant were optimized. Three background electrolytes, i.e., borate (pH 9.0), borate/phosphate (pH 8.5) and TRIS (pH 9.0) buffers, containing 50 mM SDS were investigated to separate PHT and 4HPPH. The analytes showed the best separation manner using borate buffer in terms of PHT and 4HPPH peak resolutions and peak heights. Therefore, this system was selected for further method development.

In MEKC, pH has an important influence on separation of ionizable analytes because structurally similar analytes can be separated based on even minor differences in their pKa values. To choose an appropriate pH value for the separation, the pH of the borate buffers was adjusted with 2 M NaOH to yield pH values of 7.5, 8.0, 8.5, 9.0, 9.5, 10.0, 10.5, and 11.0. The effect of pH was studied using these buffers, and the results (Table I) were investigated in terms of peak shape and the resolution between the analytes. The peak of 4HPPH had asymmetric shapes at low pH ranges, but it was improved at pH values higher than 8.5, enabling the calculation of the resolution between PHT and 4HPPH peaks. The buffer with pH 11.0 yielded better results regarding both parameters; however, due to the possibility of the bubble formation at this pH, the pH of 10.5 was selected for further investigations.

Borate concentrations of 5, 10, 15, and 20 mM were studied to optimize BGE's concentration. An increase in the concentration of the buffer improves the resolution of the analytes but leads to longer retention times. It was observed that an acceptable separation with a good repeatability was obtained using 10 mM

Table I. Effect of Buffer pH On Peak Shape and Resolution of PHT and 4HPPH

Buffer pH	4HPPH Peak shape	Resolution (min)
7.5	asymmetric	–
8.0	asymmetric	–
8.5	asymmetric	–
9.0	symmetric	1.70
9.5	symmetric	1.02
10.0	symmetric	0.85
10.5	symmetric	2.20
11.0	symmetric	3.00

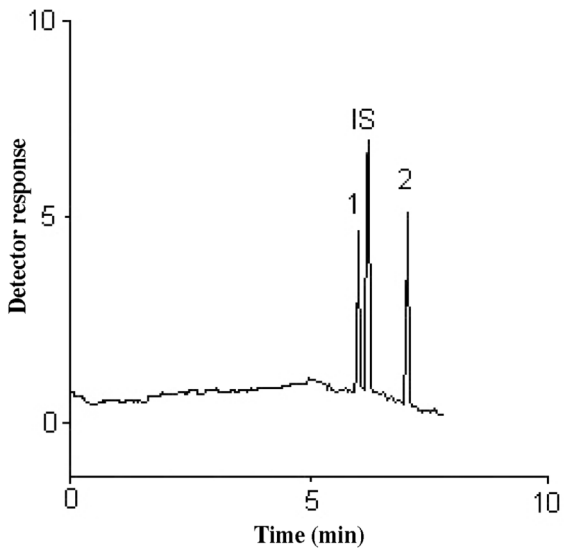

Figure 1. Electropherogram of a standard solution containing 7.5 µg/mL phenytoin, A; 5-(4-hydroxyphenyl)-5-phenylhydantoin, B; and sodium diclofenac, IS. Obtained from a capillary with the total and effective lengths of 90 and 72 cm, 50 µm i.d., detection at 214 nm, at 25ºC and borate buffer (10mM, pH 10.5) with SDS of 50 mM.

borate concentration. Therefore, this concentration of borate was used in subsequent experiments.

To study the effect of SDS concentration on the retention time and selectivity of analytes, SDS concentrations in the 20–60 mM range were tested. Addition of more SDS did not improve the resolution of the peaks but increased the retention times. A satisfactory resolution with acceptable retention time for all analytes including IS was observed using 50 mM SDS concentration.

Figure 1 shows a sample electropherogram of the standard solution of the analytes using optimum separation conditions. Separation was achieved within 8 min at 25°C by applying a constant voltage of 30 KV. This system with an optimized BGE of 10 mM borate buffer, pH 10.5, and 50 mM was selected for the determination of PHT and 4HPPH in human spiked serum. Figure 2 compares typical electropherograms obtained with the blank serum and serum spiked with PHT, 4HPPH, and IS.

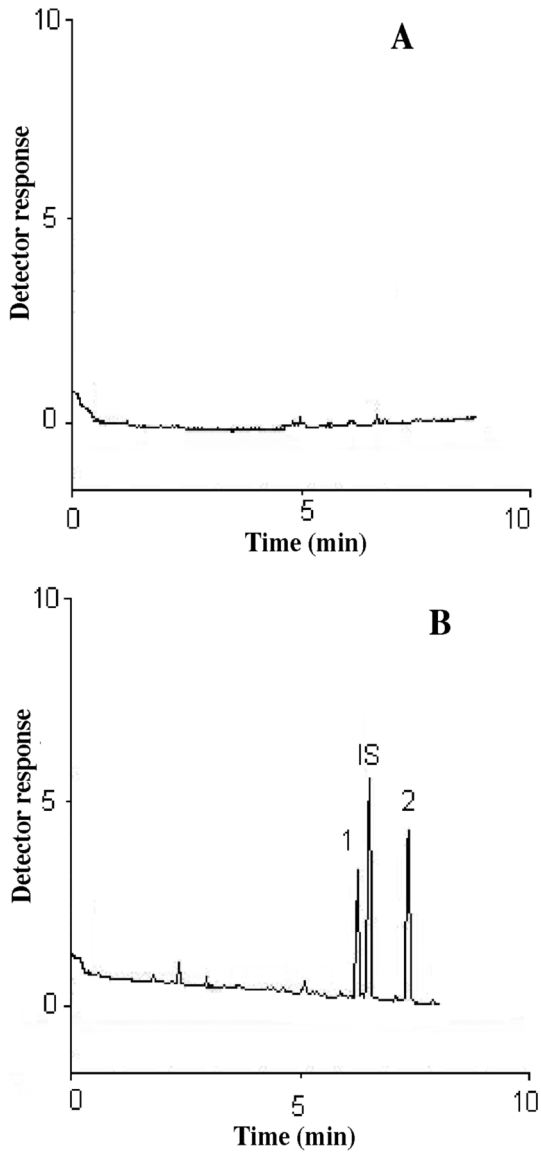

Figure 2. Electropherograms of (A) a blank serum sample and (B) blank serum sample spiked with 7.5 µg/mL PHT, 4 HPPH, and IS. The conditions are the same as in Figure 1.

Optimization of sample treatment

Both blank and spiked serum samples were subjected to a very simple and rapid liquid–liquid extraction (LLE) pretreatment procedure before analysis. The results demonstrated that this extraction procedure gave a satisfactory extraction yield and eliminated matrix interference completely, thus making it suitable for serum sample preparation.

In serum sample preparation procedure, ethyl acetate was used to precipitate serum proteins and extract analytes. Various amounts of saturated $NaHCO_3$ (100, 200, 300, 400 mL) were used in serum sample preparation to check its effect on the recovery of the analytes. The individual recoveries of PHT, 4HPPH, and IS were suitable in the sample prepared with 200 mg/mL of $NaHCO_3$ and 1 mL of ethyl acetate.

Validation of the method

Method validation was carried out with the proposed MEKC method according to the ICH guidelines (14). The calibration curves for PHT and 4HPPH were obtained in the 5–30 mg/mL concentration range by spiking blank serum with appropriate amounts of standard solution of the analytes and IS. The calibration graphs, obtained by plotting the ratio values between the area of analytes and their retention times and calculated by a least square method, were linear (r = 0.994 for PHT and r = 0.999 for 4HPPH). These results showed that a good linearity is achieved, and the method is able to determine the levels of PHT and 4HPPH in human serum over a wide concentration range. The limits of detection and quantitation were acceptable for the determination of two analytes in serum (Table II).

Intra- and inter-day precision assays were carried out to evaluate the precision of the proposed extraction and MEKC methods. Intra-day precision was calculated by performing seven consecutive injections of spiked serum samples containing 5 and

Table II. LOD and LOQ of PHT and 4HPPH in Spiked Serum*

Compound	LOD (µg/mL)	LOQ (µg/mL)
PHT	1.8	5.0
4HPPH	1.5	4.0

* LOD and LOQ = limit of detection and quantitation.

Table III. Intra-day Precision for PHT and 4HPPH in Spiked Serum

	Retention time	Peak height	Peak area
PHT: 5.0 µg/mL	0.48	3.10	1.80
PHT: 15.0 µg/mL	2.51	8.06	7.16
4HPPH: 5.0 µg/mL	0.41	1.06	2.99
4HPPP: 15.0 µg/mL	2.45	7.16	2.88

Table IV. Recovery of PHT and 4HPPH from Spiked Serum

Compound	Concentration (µg/mL)	Recovery (%)
PHT	5	88.86
	15	89.71
	25	97.35
4HPPH	5	82.17
	15	85.11
	25	89.16

15 mg/mL of each analyte, and acceptable results were obtained as shown in Table III. This procedure was also repeated over different days to obtain the interday precision. The relative standard deviations (RSDs) of corrected peak areas did not exceed 5.0% for PHT and 2.5% for 4HPPH.

In order to evaluate the individual recoveries of PHT and 4HPPH, three different concentrations of analytes were added to blank serum and analyzed after sample preparation. Recovery was calculated from peak areas of the analytes in spiked serum samples compared to peak areas of the same analyte concentration in standard solutions (Table IV). The mean extraction yield was 92.0 ± 6.3% for PHT and 85.5 ± 4.7% for 4HPPH.

Conclusion

The described MEKC method is simple and capable of separating and determining phenytoin and its main metabolite in human serum within the therapeutic range. Also, the simple and rapid sample preparation procedure permits the method to be used in routine therapeutic drug monitoring.

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