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NEW LC/MS/MS METHOD FOR THE QUANTIFICATION OF PHENYTOIN IN HUMAN PLASMA

MARCELA ACHIM[a], DANA MUNTEAN[a], LAURIAN VLASE[a], IOAN BÂLDEA[b], DAN MIHU[c], SORIN E. LEUCUȚA[a]

ABSTRACT. A simple, reversed-phase high performance liquid chromatography method with mass spectrometric detection (HPLC-MS/MS) was developed for determination of an antiepileptic drug, phenytoin, in human plasma. The procedure involves a simple extraction step by mixing 0.2 ml plasma with 0.6 ml methanol. After centrifugation, 1 µl of the supernatant was injected onto a Zorbax SB-C18 100 mm x 3 mm, 3.5 µm column, and eluted with a mobile phase consisting in a mixture of water containing 2 mM ammonium acetate and methanol 50:50 (v/v). Detection was in MRM mode, using an electrospray positive ionization. The ion transition monitored was 253.1→(182.1+225.1). The method was evaluated in terms of linearity (between 2.0 µg/ml to 80.0 µg/ml), accuracy, precision, recovery, sensitivity. The lower limit of quantification was established at 2.0 µg/ml. The simple extraction procedure and short chromatographic runtime make the method suitable for therapeutic drug monitoring studies.

Keywords: *phenytoin, HPLC-MS/MS, human plasma*

INTRODUCTION

Phenytoin (Figure 1) is widely used in the treatment of epilepsy and is effective against all types of seisures. No drug has greater need for therapeutic drug concentration monitoring and individualized dosing than phenytoin. A good correlation usually is observed between the total concentration of phanytoin in plasma and the clinical effect. Therapeutic concentration of phenytoin is above 10 µg/ml [1]. Enzyme induction by phenytoin is well documented, even auto-induction by phenytoin should be considered during the treatment with phenytoin [2].

[a] *University of Medicine and Pharmacy "Iuliu Haţieganu", Faculty of Pharmacy, Emil Isac 13, RO-400023 Cluj-Napoca, Romania, dana@tbs.ubbcluj.ro*
[b] *Babeş-Bolyai" University, Faculty of Chemistry and Chemical Engineering, Arany Janos 11, RO-400028 Cluj-Napoca, Romania*
[c] *University of Medicine and Pharmacy "Iuliu Haţieganu", Faculty of Medicine, Emil Isac 13, RO-400023 Cluj-Napoca, Romania*

Plasma concentration monitoring is widely used for the clinical management of epileptic patients receiving phenytoin [3]. To minimize toxicity, monitoring of plasma anticonvulsant levels is a part of the routine management of patients in many clinics. To the best of our knowledge, almost all of the methods that were applied for determination of antieplileptic drugs in biological media are often chromatography, electrophoresis and immunoassay techniques [4,5].

The aim of the present study was to develop a fast LC-MS/MS method, able to quantify phenytoin in human plasma after a simple sample preparation by protein precipitation. The proposed method proved to be accurate and despite of very simple sample preparation, showed high sensitivity.

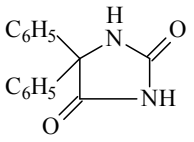

Figure 1. Molecular structure of phenytoin

RESULTS AND DISCUSSION

Figure 2 shows representative chromatograms of drug-free (blank) human plasma and a sample containing 2.0 µg/ml phenytoin (LOQ). No significant interference at the retention time of phenytoin (1.6 min) was observed, due to the specificity of the selected signal (Figure 3).

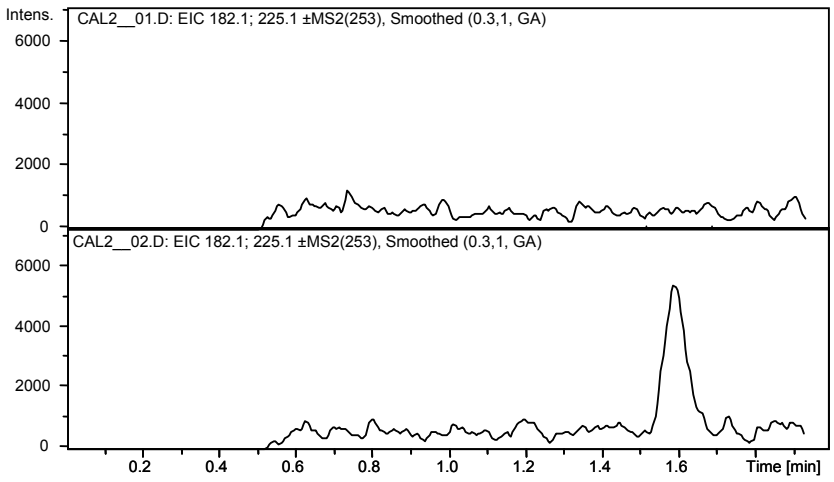

Figure 2. Chromatograms of a drug-free plasma sample (up) and LOQ plasma standard with 2.0 µg/ml phenytoin (down)

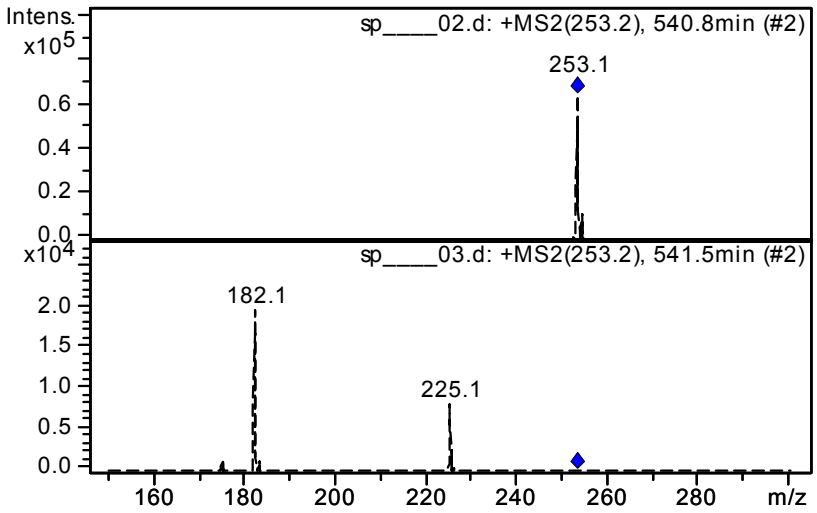

Figure 3. Mass spectra of phenytoin. Full-scan spectrum (up) and MS/MS spectrum (down). The sum of ions with *m/z* 182.1 and 225.1 was used for quantification

The analyte detection was made in MRM mode, the ion transition monitored was *m/z* 253.1→(*m/z* 182.1 + *m/z* 225.1). This way, the detection is more sensitive than in case based only on ion *m/z* 253.1 (Figure 3).

The analyte carryover was verified using a blank injection made right after an injection of the most elevated concentration level from calibration curve. No interference at retention time of analyte due to carryover was observed.

The mean calibration curve y = a(±S.D.)x + b(±S.D.), with S.D. standard deviation, was: y = (59590.4±1971.9)x – (10490.5±2501.6), N = 8 calibration points, n = 5 determinations for each calibration point. The residuals had no tendency of variation with concentration. The applied calibration curve model proved to be accurate over the concentration range 2 – 80 µg/ml, with a correlation coefficient grater than 0.998 (Figure 4).

The intra-day precision was determined from replicate analysis of samples containing phanytoin at four different concentrations covering the low, medium and higher ranges of calibration curve (Table 1), in good agreement to international regulations regarding bioanalytical methods validation [6-8]. The intra-day precision and accuracy ranged from 1.9% to 9.5% and 3.7% to 10% respectively.

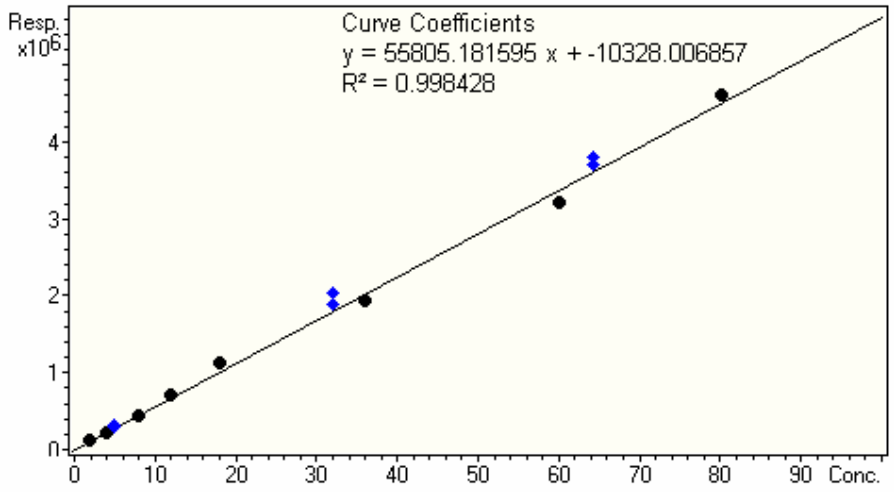

Figure 4. Calibration curve for phenytoin

Table 1. Intra-day precision, accuracy and recovery for phenytoin (n = 5)

$c_{nominal}$ (µg/ml)	Mean c_{found} (µg/ml) (± S.D.)	C.V. %	Bias %	Recovery % (± S.D.)
2.0	2.07 (0.2)	9.5	3.7	106.5 (10.5)
5.0	5.50 (0.10)	1.9	10.0	100.3 (1.9)
32.0	34.87 (1.94)	5.6	9.0	102.4 (5.7)
64.0	67.60 (3.74)	5.5	5.6	101.1 (5.6)

The intra-day precision and accuracy were determined by analyzing in five different days samples that have the same concentration, at lower, medium and higher levels from calibration curve. The precision ranged from 3.5% to 10.1% and accuracy from 1.5% to 7.2 % (Table 2):

Table 2. Inter-day precision, accuracy and recovery for phenytoin (n = 5)

$c_{nominal}$ (µg/ml)	Mean c_{found} (µg/ml) (± S.D.)	C.V. %	Bias %	Recovery % (± S.D.)
2.0	2.03 (0.20)	10.1	1.5	121.1 (35.3)
5.0	5.36 (0.21)	4.0	7.2	97.3 (9.1)
32.0	33.10 (1.16)	3.5	3.4	101.4 (5.6)
64.0	66.32 (2.87)	4.3	3.6	99.1 (4.6)

Under the experimental conditions used, the lower limit of quantification (LOQ) was of 2 µg/ml phenytoin. LOQ is the lowest amount of analyte which can be measured with accuracy and precision less than 20%.

CONCLUSIONS

A simple, sensitive, accurate and precise HPLC/MS/MS method for determination of phenytoin in human plasma using a simple single-step extraction procedure is reported. Another advantage of the method is the short chromatographic runtime of only 2.1 min. The method is suitable for therapeutic drug monitoring studies and can also be used for pharmacokinetic studies conducted on healthy volunteers [9-13].

EXPERIMENTAL SECTION

Reagents

Phenytoin, methanol, ammonium acetate, were purchased from Merck (Merck KgaA, Darmstadt, Germany). Solvents used were HPLC grade and all other chemicals were of analytical grade. Distilled, deionised water was produced by a Direct Q-5 Millipore (Millipore SA, Molsheim, France) water system. The drug-free human plasma was supplied by the Local Bleeding Centre Cluj-Napoca, Romania.

Preparation of standard solutions

A stock solution containing 10 mg/ml phenytoin was prepared in methanol. A working solution of 200 µg/ml was prepared by diluting the appropriate volume of stock solution with plasma. Than this was used to spike different volumes of drug-free plasma, providing finally eight plasma standards with concentration between 2.0 and 80.0 µg/ml. Quality control samples (QC) of 5.0, 32.0, and 64.0 µg/ml were prepared by diluting specific volumes of working solution with plasma and were used to evaluate precision and accuracy of the method.

Chromatographic and mass spectrometry systems and conditions

The HPLC system was an 1100 series model (Agilent Technologies) consisted in a binary pump, an in-line degasser, an autosampler, a column thermostat and an Ion Trap VL mass spectrometer detector (Bruckner Daltonics GmbH, Germany). Chromatograms were processed using QuantAnalysis Software. The detection of phenytoin was in MRM (MS/MS) mode, using an electrospray positive ionization (ESI positive). The ion transitions monitored was: *m/z* 253.1→(*m/z* 182.1 + *m/z* 225.1). Chromatographic separation was performed at 45ºC on a Zorbax SB-C18 100 mm x 3 mm, 3.5 µm column (Agilent Technologies), protected by an inline filter.

Mobile phase

The mobile phase consisted in a mixture of water containing 2 mM ammonium acetate and methanol (50:50 v/v). It was always freshly prepared and was degassed before elution for 10 min in am Elma Transsonic 700/H (Singen, Germany) ultrasonic bath. The pump delivered the mobile phase at a flow rate of 1 ml/min.

Sample preparation

Plasma samples were prepared as follows in order to be chromatographically analyzed: in an Eppendorf tube (max 1.5 ml), 0.2 ml plasma and 0.6 ml methanol were added. The tube was vortex-mixed for 10 s (Vortex Genie 2, Scientific Industries) and centrifuged for 6 min at 5000 rpm (2-16 Sartorius centrifuge, Osterode am Harz, Germany). The supernatant was transferred to an autosampler vial and 1 µl was injected into the HPLC system.

Validation

As a first step of method validation [6-8], specificity was verified using six different plasma blanks obtained from healthy volunteers who had not previously taken any medication.

The concentration of the analyte was determined automatically by the instrument data system. The calibration curve model was $y = ax + b$, weight $1/y$ linear response, where y-peak area and x-concentration. Distribution of the residuals (% difference of the back-calculated concentration from the nominal concentration) was investigated. The calibration model was accepted, if the residuals were within ±20% at the lower limit of quantification (LOQ) and within ±15% at all other calibration levels and at least 2/3 of the standards meet this criterion, including highest and lowest calibration levels.

The intra-day and inter-day precision (expressed as coefficient of variation, CV%) and accuracy (expressed as relative difference between obtained and theoretical concentration, bias%) of the assay procedure were determined by analysing on the same day five different samples at each of the lower (5.0 µg/ml), medium (32.0 µg/ml) and higher (64.0 µg/ml) levels of the considered concentration range and one different sample of each at five different occasions, respectively.

The recovery of phenytoin was analyzed at each of the three concentration levels mentioned above, e.g. lower, medium and higher level, and also at the quantification limit, by comparing the peack area response of spiked plasma samples with the response of standards prepared in water with the same concentration of ivabradine as the plasma samples, all these prepared as stated in section “Sample preparation”.

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