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QUANTIFICATION OF PHENOBARBITAL IN HUMAN PLASMA BY LC/MS/MS FOR THERAPEUTIC DRUG MONITORING

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ABSTRACT. A simple and sensitive LC/MS/MS method for quantification of phenobarbital and in human plasma has been developed and validated. Phenobarbital was separated under isocratic conditions using a mobile phase of 70:30 (v/v) 0.1% acetic acid and acetonitrile. In these chromatographic conditions, the retention time of phenobarbital was 1.2 min and the overall time of one analysis was 1.7 min. Plasma sample preparation consisted in protein precipitation with methanol. The detection of phenobarbital was realized in MRM mode using an ion trap mass spectrometer with electrospray negative ionization. The linearity domain was established between 2.00 and 80.00 µg/mL. Accuracy (bias%) and precision (CV%) were less than 9.8% for intra-day assay and 12.6% for inter-day assay. The recovery ranged between 95.9 and 136.4%. The method is very simple and fast and was used for therapeutic drug monitoring of phenobarbital.

Keywords: *phenobarbital, LC/MS/MS, therapeutic drug monitoring*

INTRODUCTION

Phenobarbital, 5-Ethyl-5-phenyl-2,4,6-pyrimidinetrione, is widely used for his properties as anesthetic, sedative, hypnotic and anticonvulsant drug (Fig. 1). After oral administration phenobarbital presents 95% bioavailability, with a very long half life of 72 to 144 hours. Phenobarbital is metabolized by the liver, mainly through hydroxylation and glucuronidation and it is excreted primarily by the kidneys. The currently accepted therapeutic concentration range for phenobarbital in plasma is 10 to 30 µg/mL [1].

Being a drug with large inter-subject variability and narrow therapeutic window, therapeutic drug monitoring is required for phenobarbital in order to improve its pharmacotherapy and safety.

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Several methods for determination of phenobarbital concentration in human plasma have been reported. Mainly, high performance liquid chromatograpy (HPLC) methods with UV detection were described [2,4,5], Liquid chromatography coupled with mass spectrometry (LC/MS or LC/MS/MS) methods were reported also [3]. LC/MS has been widely accepted as the most used method in the identification and quantitative analysis of drugs and its metabolites because of its superior sensitivity and specificity.

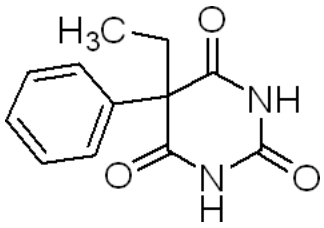

Figure 1. Molecular structure of phenobarbital

The aim of present study is the development of a rapid and specific LC/MS/MS method for the quantification of phenobarbital in human plasma in order to be applied in therapeutic drug monitoring or pharmacokinetic studies. In comparison with previously published HPLC methods (Table 1), the proposed method has the advantage of simple sample preparation by protein precipitation and a very short time of analysis.

RESULTS AND DISCUSSION

In the electric field created by the electrospray ionization source, the molecule of phenobarbital readily loose an proton, an negative ion being generated, with m/z 231 (Fig. 2). Thus, the detection of phenobarbital was carried out in multiple reaction monitoring (MRM) mode, by fragmentation of the parent ion with the m/z 231. The fragmentation of the parent ion is induced by collision of the ion with helium in the ion trap and a stable product ion with m/z 188 is observed in the mass spectra (Fig. 2). Finally, the abundance of ion with m/z 188 was monitored and quantitatively correlated with phenobarbital concentration. The retention time of phenobarbital was 1.2 min (Fig. 3) and, due detection specificity, no significant interference was observed at the retention time in plasma blank samples chromatograms.

Table 1. Analytical characteristics of several reported HPLC or LC/MS methods for the determination of phenobarbital in plasma

References	Matrix	Detection	Mobile phase constituents	Extraction	LOQ (µg/mL)	Run time (min)
Paibir et al. [2]	Human urine	HPLC-UV	12:88 (v/v) acetonitrile-H_3PO_4 (pH 2.3; 0.025 M)	Filtration	0.7	50
Kanazawa et al. [3]	Human plasma	LC-APCI-MS	30:70 (v/v) acetonitrile–0.1 M potassium phosphate buffer	Solid phase extraction	n/a	8
Moriyama et al. [4]	Rat offspring plasma	HPLC-UV	25:75 (v/v) acetonitrile-0.01 M KH_2PO_4	Solid phase extraction	5	10
Costa Q et al. [5]	Human plasma	HPLC-UV	22:78 (v/v) acetonitrile-water	Stir bar-sorptive extraction	0.08	5

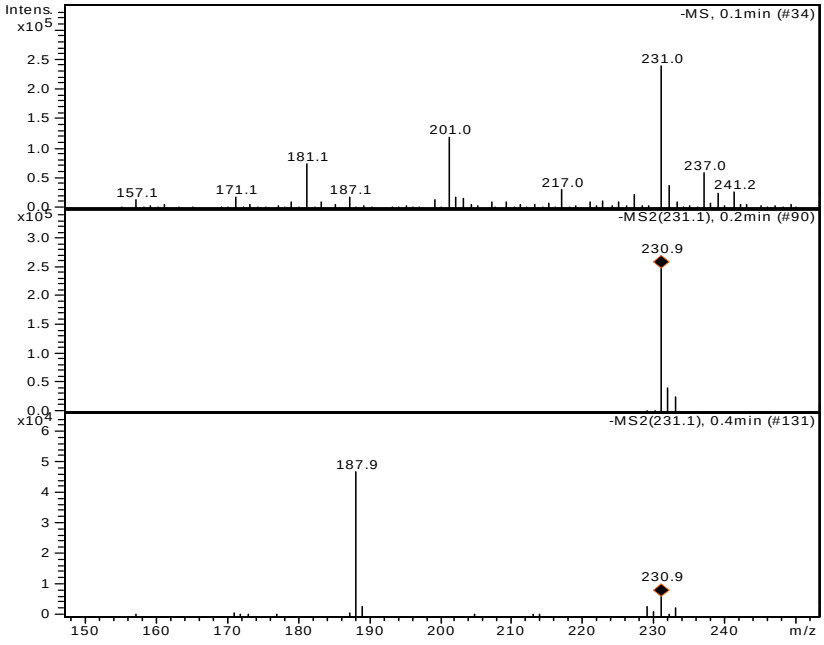

Figure 2. Ion mass spectra used for detection and quantification of phenobarbital from human plasma (upper spectra - full scan, middle spectra - isolation, lower spectra - fragmentation).

The calibration curves showed linear response over the range of concentration used in the assay procedure. The calibration curve for phenobarbital was in the concentration range 2.00-80.00 µg/mL, using 7 calibration levels, n = 5 days, with a coefficient of correlation greater than 0.997. The residuals had no tendency of variation with concentration and were between ±14.6% values.

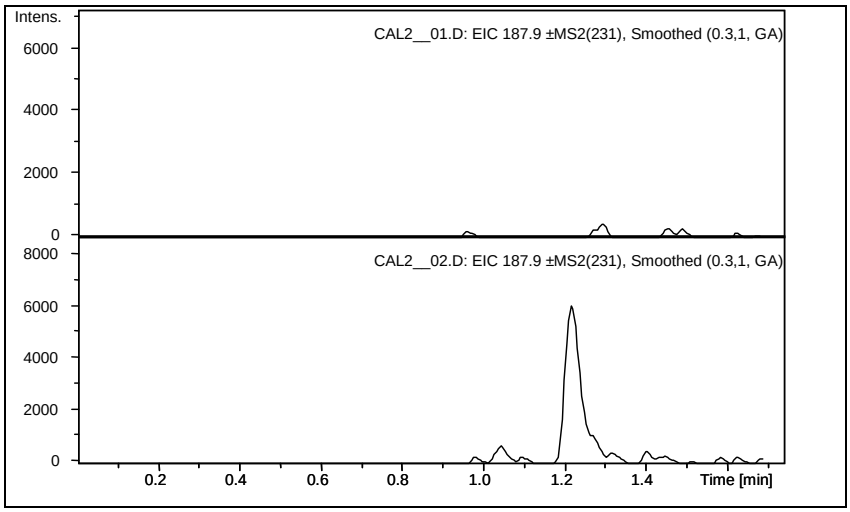

Figure 3. Typical chromatogram of the LLOQ plasma standard with 2.00 µg/mL phenobarbital

The inter- and intra-day precision and accuracy results are showed in Table 2 and Table 3 and they are in agreement to international regulations regarding bioanalytical methods validation [6-11]. Precision and accuracy were from –6.7% to 9.8% for intra-day assay and from -1.2% to 12.6% for inter-day assay. The lower limit of quantification was established at 2.00 µg/mL phenobarbital. Precision and accuracy at quantification limit were 15.4% and -17.8% for intra-day determinations and 11.4% and 6.8% for inter-day determinations, respectively. The recovery was consistent and ranged between 95.9 and 136.4% (Tables 2 and 3).

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

$c_{nominal}$ µg/mL	Mean c_{found} µg/mL (± S.D.)	CV %	Bias %	Recovery % (± S.D.)
2.00	1.64±0.25	15.4	-17.8	95.9±19.2
5.00	5.12±0.50	9.8	2.4	98.4±8.5
32.00	32.88±2.23	6.8	2.7	102.4±6.8
64.00	59.69±1.24	2.1	-6.7	101.0±2.1

Table 3. Inter-day precision, accuracy and recovery (n = 5) for phenobarbital

$c_{nominal}$ µg/mL	Mean c_{found} µg/mL (± S.D.)	CV %	Bias %	Recovery % (± S.D.)
2.00	2.14±0.24	11.4	6.8	136.4±16.0
5.00	5.50±0.69	12.6	10.1	100.5±9.0
32.00	32.70±2.69	8.2	2.2	98.1±3.6
64.00	63.22±2.17	3.4	-1.2	99.0±4.2

CONCLUSIONS

The proposed method provides accuracy and precision for quantitative determination of phenobarbital in human plasma. The simple sample preparation by protein precipitation, the selected MS transition for monitoring and a very short time of chromatographic analysis allows a specific and efficient determination of a large number of plasma samples in a short time. This high-throughput method is suitable for therapeutic drug monitoring or pharmacokinetic investigations of phenobarbital.

EXPERIMENTAL SECTION

Reagents

Phenobarbital was reference standard from Sigma-Aldrich (Sigma-Aldrich Chemie Gmbh, Munich, Germany). Acetonitril, acetic acid and methanol were Merck products (Merck KgaA, Darmstadt, Germany). Distilled, deionised water was produced by a Direct Q-5 Millipore (Millipore SA, Molsheim, France) water system. The human blank plasma was supplied by the Local Bleeding Centre Cluj-Napoca, Romania.

Standard solutions

A stock solution of phenobarbital with concentration of 10 mg/mL was prepared by dissolving appropriate quantity of reference substance in 10 mL acetonitril. A working solution was obtained by diluting a specific volume of stock solution with plasma. Then this was used to spike different volumes of plasma blank, providing finally seven plasma standards with the concentrations ranged between 2.00 and 80.00 µg/mL. Accuracy and precision of the method was verified using plasma standards with concentrations of 2.00, 5.00, 32.00 and 64.00 µg/mL phenobarbital.

Chromatographic and mass spectrometry systems and conditions

The HPLC system was an 1100 series model (Agilent Technologies) consisted of a binary pump, an in-line degasser, an autosampler, a column thermostat, and an Ion Trap VL mass spectrometer detector (Brucker Daltonics GmbH, Germany). Chromatograms were processed using QuantAnalysis software. The detection of phenobarbital was MS/MS using an electrospray

negative ionisation (ESI negative). The monitored ion transition was from the m/z 231.1 ion to m/z 187.9 ion. Chromatographic separation was performed at 45 ◦C on a Zorbax SB-C18 100 mm x 3 mm i.d., 3.5 µm column (Agilent Technologies), protected by an in-line filter.

Mobile phase

The mobile phase consisted of a mixture of 0.1% acetic acid and acetonitrile (70:30 (v/v), each component being degassed, before elution, for 10 min in an Elma Transsonic 700/H (Singen, Germany) ultrasonic bath. The pump delivered the mobile phase at 1 mL/min.

Sample preparation

Standard and test plasma samples were prepared as follows in order to be chromatographically analyzed. In an Eppendorf tube 0.2 mL plasma were mixed with 0.6 mL methanol. The tube is vortex-mixed for 10 s and then centrifuged for 6 min at 5000 rpm. A volume of 0.15 mL of supernatant is transferred in an autosampler vial and 1 µl was injected into the HPLC system.

Method validation

Method validation involves verifying specificity [6,7], by using six different plasma blanks obtained from healthy human volunteers who did not take before phenobarbital and any other medication. The linearity of the peak area against standard concentration was verified between 2.00-80.00 µg/mL phenobarbital by applying least-squares linear regression. The applied calibration model was: $y = a*x+b$, 1/y weight, where y is 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 and within ±15% at all other calibration levels and at least 2/3 of the standards meet this criterion.

The limit of quantification was established as the lowest calibration standard with an accuracy and precision less than 20%. The intra- and inter-day precision (expressed as coefficient of variation %, CV%) and accuracy (relative difference % between found and theoretical concentration, bias%) of the assay procedure were determined by the analysis in the same day of three samples at each of three levels of concentration in the considered concentration range and one sample of each in three different days, respectively. The recoveries at each of previous levels of concentration were measured by comparing the response of the treated plasma standards with the response of standards in water with the same concentration in phenobarbital as the final extract from plasma standards.

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