



A Novel HPLC Method for Determination of Phenytoin in Human Plasma

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Authors' contributions

This work was carried out in collaboration between all authors. Author JF conducted validation of the HPLC method and wrote the first draft of the manuscript. Author SA developed the analytical method. Author MB managed the the study. All authors read and approved the final manuscript.

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ABSTRACT

Aim: Aim of this research was to develop and validate a simple, efficient and reproducible high-performance liquid chromatography method to measure phenytoin concentrations in human plasma

Study Design: Linearity, selectivity, sensitivity, accuracy and precision of the analytical methods were validated according to ICH guidelines.

Methodology: The method employed a Phenomenex C₁₈ column kept at 25°C. The mobile phase consisted of a 0.05 M potassium dihydrogen phosphate buffer solution (pH 2.8) and methanol in a ratio of 60:40, respectively. The flow rate of the mobile phase was 0.7 mL/min. Phenytoin was detected at a wavelength of 250 nm.

Results: Phenytoin was eluted at 7.4 minutes with no interference with the other components of the human plasma. The method was linear in the range of 1-25 µg/mL (R² = 0.9998). The LOD and LOQ were calculated as 0.07 µg/mL and 0.20µg/mL, respectively. Recovery, tested in the range of 5-20 µg/mL, was found to be 99.21 - 102.09%. Intraday and interday precision RSDs were 0.65 and

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0.29%, respectively.

Conclusion: The proposed method is fast, reliable and reproducible, and can be recommended to measure free phenytoin levels in the blood.

Keywords: Phenytoin; HPLC; validation; human plasma.

1. INTRODUCTION

Phenytoin (PHT) is an anticonvulsant medication marketed in the U.S. as Dilantin™. This drug has been indicated for the management of generalized tonic-clonic seizures, complex partial seizures, and for the prevention of seizures after head trauma and neurosurgery [1,2]. There is interest in examining the activity of the drug due to its narrow therapeutic index, nonlinear pharmacokinetics and the ability of various compounds to modify the concentrations of phenytoin in the blood [2-5]. Drugs in the body exist in two forms bound and unbound. Only unbound (free) drug reaches the active site, interacts with receptors and produces a therapeutic effect. Free drug is a predictor of clinical efficacy or toxicity [6-8]. PHT is approximately 90% bound to plasma proteins [9-11]. Changes in unbound concentrations of PHT can lead to exaggerated pharmacologic response and toxicity. The development of side effects also correlates better with free phenytoin concentrations than with total concentrations [12-14]. PHT, as a drug with nonlinear pharmacokinetics and narrow therapeutic index, may produce major changes in the pharmacological effect with the only small variations in its unbound plasma concentrations. Monitoring of free PHT plasma levels is required to avoid unexpected alterations in pharmacologic response, to achieve the efficacy of phenytoin and to avoid phenytoin intoxication. Unbound phenytoin plasma concentrations should be measured in patients rather than calculated [15]. Our study aimed to develop a simple, efficient and reproducible high-performance liquid chromatography (HPLC) method to assess unbound PHT levels in human plasma. Previous studies have measured PHT levels using HPLC methods outside plasma [16,17], involved solid-phase extraction protocols [18] as well as detected PHT concentrations in biological fluids [19-25]. This novel HPLC method was used to assess free plasma concentrations of phenytoin in drug-drug interaction studies [26].

2. MATERIALS AND METHODS

2.1 Chemicals and Reagents

Phenytoin sodium (5,5-Diphenylhydantoin sodium salt), dimethyl sulfoxide (DMSO), HPLC-grade methanol, HPLC-grade water and 0.22 µm Stericup™ Sterile Vacuum Filter Units were obtained from Fisher Scientific (New Hampshire, USA). Potassium dihydrogen phosphate and Centrifree® Ultrafiltration Devices were purchased from EMD Millipore (Massachusetts, USA). Human plasma was obtained from Valley Biomedical (Virginia, USA).

2.2 Instrument and Chromatographic Conditions

An Agilent 1100 series HPLC system with autosampler and UV detector was used for the study. A Phenomenex Kinetex C₁₈ LC column (250 x 4.6 mm) was operated at 25°C. An isocratic method was utilized to elute the analyte with a mobile phase flow rate of 0.7 mL/min. The injection volume was 20 µL for all samples. Phenytoin was detected at a wavelength of 250 nm.

2.3 Mobile Phase Preparation

The mobile phase consisted of a 0.05 M potassium dihydrogen phosphate (KH₂PO₄) buffer solution (pH adjusted to 2.8 with phosphoric acid) and methanol (MeOH) in a 60:40, MeOH:KH₂PO₄ ratio. The mobile phase was filtered using a 0.22 µm Stericup™ Sterile Vacuum Filter Unit and degassed by a vacuum stirring setup.

2.4 Stock Solution and Samples Preparation

Phenytoin was dissolved in HPLC-grade DMSO to produce a stock solution of 1 mg/mL concentration. The master stock was diluted with filtered human plasma to produce another stock solution of 100 µg/mL, which was further diluted to achieve required concentrations of phenytoin.

Human plasma was passed through Millipore ultracentrifugation units, which were run at 2,000X g for 20 minutes. The resulting filtrate was collected and used as a diluent for the PHT stock solution.

3. METHOD VALIDATION

The analytical method was validated based on ICH Harmonised Tripartite Guideline [27].

3.1 Specificity

The specificity of the method was determined by comparing blank (plasma without PHT) chromatogram to the chromatogram of a sample containing a known concentration of PHT. Analysis of the sample containing PHT showed a

sharp peak at an elution time of 7.4 min, which was absent on the blank chromatogram (Figure 1). Other components of filtered plasma were detected and separated by the method, allowing proper visualization of the PHT peak. The experiments were performed in triplicate.

3.2 Linearity

To determine linearity, eight phenytoin concentrations ranging from 1 to 25 µg/mL were prepared from the stock solution. Filtered human plasma was used to dilute the PHT stock solution into 1, 2, 4, 6, 8, 10, 15 and 25 µg/mL concentrations. The samples were assessed to HPLC. The mean area for each concentration was plotted against the concentration (Fig. 2).

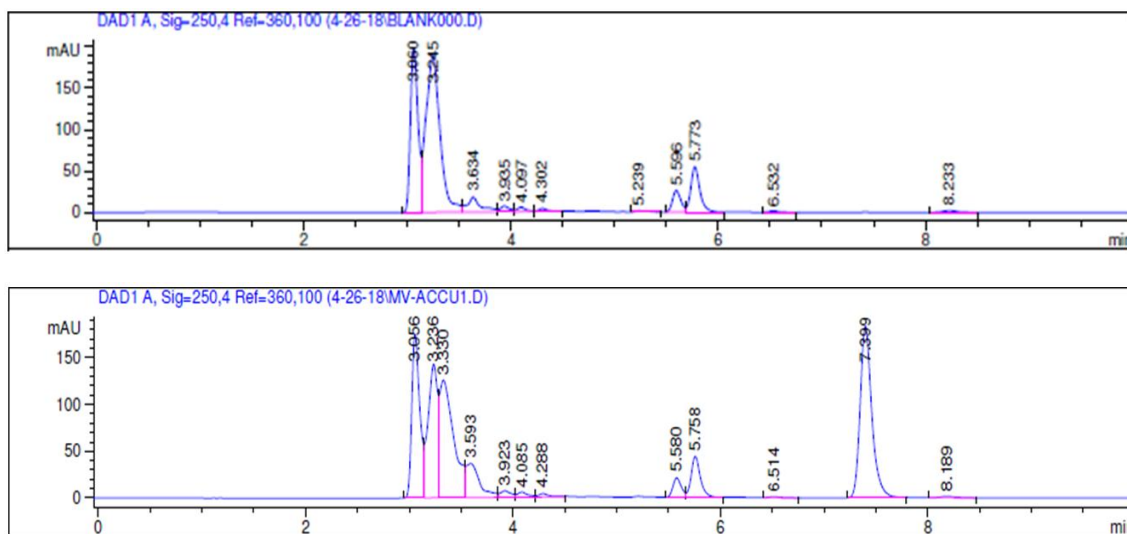


Fig. 1. Blank Chromatogram (top) and Phenytoin Chromatogram (bottom): PHT concentration is 25 µg/mL, retention time is 7.399 minutes

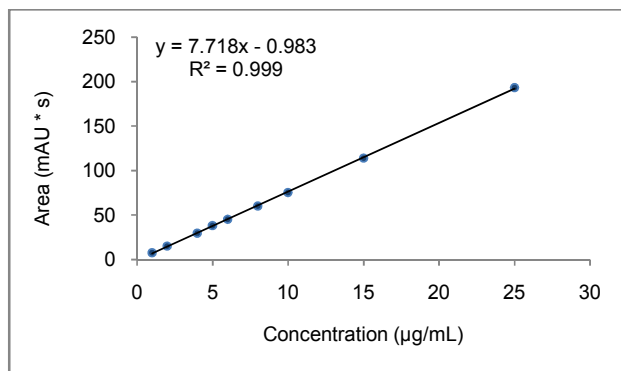


Fig. 2. The linearity of the HPLC method

An excellent correlation was detected between the peak areas and concentrations tested ($R^2 = 0.9998$). All experiments were performed in triplicate.

3.3 Limit of Detection and Limit of Quantitation

The limit of detection (LOD) and limit of quantitation (LOQ) were calculated based on ICH guidelines [11]. The following formulas were used:

$$\text{LOD} = 3.3 \cdot \sigma / S$$

$$\text{LOQ} = 10 \cdot \sigma / S$$

Where:

- σ- refers to the standard deviation of the y-intercept of the calibration curve
- S- refers to the slope of the calibration curve.

The calculated LOD and LOQ were at 0.07 µg/mL and 0.20 µg/mL, respectively (Table 1).

3.4 Accuracy

Accuracy study was performed with PHT concentrations of 5 µg/mL, 10 µg/mL and 20

µg/mL. Triplicates were made to assess the accuracy. The results of the study are summarized in Table 2. The mean % recoveries of the three concentrations were found in the range of 99.21 - 102.09%.

Table 1. LOD and LOQ of the HPLC method

σ	0.16
S	7.71
Limit of Detection (µg/mL)	0.07
Limit of Quantitation (µg/mL)	0.20

3.5 Precision / Repeatability

Precision was determined by utilizing inter- and intraday testing. Phenytoin concentration of 25 µg/ml was used in both studies. In the intraday study, six injections of different samples of 25 µg/mL concentration were evaluated. In the interday study, an injection of six different samples of the same concentration (25 µg/mL) was done once a day for six consecutive days. The results of the study are summarized in Table 3. The results have been demonstrated low relative standard deviations (RSD) that indicate a precise method [28].

Table 2. Accuracy of the HPLC Method

Sample	PHT concentration (µg/mL)	Measured PHT Concentration(µg/mL)	% Recovery	Mean % Recovery
1	5	5.01	100.28	100.55
		5.03	100.67	
		5.03	100.68	
2	10	9.93	99.27	99.21
		9.91	99.15	
		9.92	99.22	
3	20	20.26	101.28	102.09
		20.63	103.17	
		20.36	101.81	

Table 3. Precision of the HPLC method

Sample	Intraday Concentration(µg/mL)	Interday Concentration(µg/mL)	Day
1	25.15	25.13	1
2	24.90	25.14	2
3	24.80	25.03	3
4	24.85	25.16	4
5	25.25	25.01	5
6	25.03	24.97	6
Mean	24.99	25.07	
SD*	0.16	0.07	
%RSD**	0.65	0.29	

*SD is Standard Deviation

**RSD is Relative Standard Deviation

Table 4. Stability of phenytoin

Initial PHT concentration (µg/mL)	Stability conditions				
	Human plasma			Mobile phase	
	RT, 4h	4°C, 24 h	-30°C, 7days	Freeze-Thaw	4°C 24 h
PHT concentrations (µg/mL)					
5	5.06	5.01	5.41	5.01	4.99
25	25.17	25.19	25.13	25.33	25.34

3.6 Stability

The stability of phenytoin in human plasma was evaluated and the results are reported in Table 4.

All the samples demonstrated stability for up to 4 h at room temperature (RT), for 24 h at 4°C, for 7 days at -30°C and after three freeze-thaw cycles.

4. CONCLUSION

The goal of the study was to develop and validate a simple and effective HPLC method to measure unbound phenytoin concentrations in human plasma. The analytical technique was fully validated. The novel HPLC method demonstrated phenytoin sharp peak, good resolution and no interferences from plasma constituents. The analytical procedure is a simple, fast, selective, precise and accurate method for quantitation of phenytoin in human plasma and may be utilized in studies where free phenytoin levels need to be measured. Detecting of free PHT plasma levels should be conducted to escape variations in pharmacologic response, to achieve the efficacy of phenytoin and to avoid phenytoin intoxication novel HPLC method has the necessary sensitivity to be used clinically for TDM (therapeutic drug monitoring) as well as for pharmacokinetics and drug: drug interaction studies. The this newly developed analytical technique has been applied for the drug: drug interaction studies between phenytoin and tizoxanide [26].

CONSENT

It is not applicable.

ETHICAL APPROVAL

It is not applicable.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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