ELSEVIER

Journal of Pharmaceutical and Biomedical Analysis
23 (2000) 573–579

JOURNAL OF
PHARMACEUTICAL
AND BIOMEDICAL
ANALYSIS

www.elsevier.com/locate/jpba

# Determination of free levels of phenytoin in human plasma by liquid chromatography/tandem mass spectrometry

Sofiya Bardin, James C. Ottinger, Alan P. Breau, Thomas J. O'Shea *

*Searle, 4901 Searle Parkway, Skokie, IL 60077, USA*

Accepted 23 March 2000

**Abstract**

A liquid chromatography combined with tandem mass spectrometry assay for the determination of free levels of the highly protein bound drug phenytoin (5,5-diphenylhydantoin) in human plasma is described. The assay was demonstrated to be reliable, accurate and precise, and specific for phenytoin. The procedure involves isolation of the unbound drug from the drug/protein complex by ultrafiltration. Liquid–liquid extraction was employed to extract the resultant ultrafiltrate. PHT was separated on a $50 \times 3$ mm reversed-phase column using isocratic mobile phase conditions that yielded a run time of 1.5 min, enabling high throughput sample analysis. Linearity was obtained over the range 5.00 to 500 ng/ml. Both between-run and within-run coefficients of variation were less than 15% and accuracy's across the assay range were all within $100 \pm 10\%$. The assay was successfully implemented to support a clinical interaction study with phenytoin. 

*Keywords:* Phenytoin; Protein binding; Human plasma; Ultrafiltration; Reversed-phase chromatography; Tandem mass spectrometry

## 1. Introduction

The extent of drug binding to plasma proteins varies widely among different drugs. The effects of changes in the extent of protein binding on the unbound drug fraction and on drug metabolism are significant only for highly bound drugs ($>$ 80% bound), as a relatively small change in the degree of binding has a significant effect on the level of unbound drug [1]. As only the free fraction of the drug is pharmacologically active, the determination of free levels is an important factor in establishing the pharmacokinetic and pharmacodynamic properties of a drug.

Phenytoin (5,5-diphenylhydantoin) one of the most commonly prescribed anti-convulsant drugs for the treatment of epilepsy, has reported protein binding levels up to 90% [2]. Phenytoin has a narrow therapeutic range (1–2 μg/ml free drug) combined with a low threshold ($>2$ μg/ml free drug) for toxicity that necessitates therapeutic monitoring of the free concentration of the drug to ensure efficacy and avoid toxicity. Protein binding of this drug can be affected by a range of factors including hypoalbuminemia, renal dis-

* Corresponding author. Tel.: +1-847-9828136; fax: +1-847-9824857.
*E-mail address:* thomas.j.oshea@monsanto.com (T.J. O'Shea).

0731-7085/00/$ - see front matter 
PII: S0731-7085(00)00338-1

eases, age and displacement by other drugs sharing the same binding site on albumin. Also, drug interactions (induction and inhibition) for several agents that are metabolized by the same cytochrome P-450 isozymes as phenytoin have been documented [3].

Reported literature approaches for this analysis include separation of free phenytoin by equilibrium dialysis and analysis by high performance liquid chromatography (HPLC) with UV detection [4], ultrafiltration followed by HPLC with UV detection [5], and automated sequential trace enrichment of dilalysate (ASTED) with HPLC with UV detection [6]. These approaches all have associated disadvantages including long sample preparation time and analysis time, and high limits of quantitation.

The goal of this study was to develop and validate an assay that would improve the speed, sensitivity and simplicity of reported methods for the determination of free concentrations of phenytoin in human plasma. The assay was developed to support clinical interaction studies with phenytoin.

## 2. Experimental

### 2.1. Reagents

Phenytoin (PHT) (Fig. 1) and ammonium acetate ACS reagent grade was obtained from Aldrich Chemical (Milwaukee, WI). Pentobarbital (PB) (Fig. 1) was purchased from Sigma (St. Louis, MO). HPLC grade methanol and acetonitrile were obtained from Baxter Healthcare, Burdick and Jackson Division (Muskegon, MI). Ammonium hydroxide and methyl-*tert*-butyl ether were purchased from J.T. Baker (Phillipsburg, NJ). All other chemicals were analytical reagent grade. Water was deionized and filtered using a Millipore Milli-Q UV plus system.

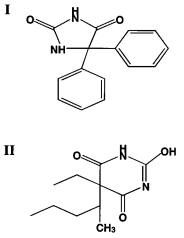


Fig. 1. The chemical structures of phenytoin (**I**) and the internal standard, pentobarbital (**II**).

### 2.2. Instrumentation

Liquid chromatography with tandem mass spectrometry detection (LC/MS/MS) was performed on a Perkin-Elmer Sciex (ON, Canada) model API III$^+$ tandem triple quadruple mass spectrometer equipped with a heated pneumatic nebulizer interfaced to a chromatographic system comprising of a Hewlett-Packard (Paula Alto, CA, USA) 1050 quaternary pump and a Perkin-Elmer (CA, USA) model ISS 200 autosampler. The data were processed using MacQuan (version 5.0) software (PE-Sciex) on a Macintosh Quadra 650 computer.

### 2.3. Chromatographic conditions

Isocratic chromatographic conditions were employed using a an Inertsil ODS-3 ($50 \times 3$ mm, 5 μm) analytical column coupled to an Inertsil ODS-3 guard cartridge ($20 \times 2$ mm, 5 μm). The mobile phase used for analysis was a mixture of acetonitrile:5 mM ammonium buffer, 60:40, v/v. The ammonium buffer was prepared using ammonium acetate and ammonium hydroxide. The mobile phase was delivered at a flow rate of 0.7 ml/min. The retention times of phenytoin and pentobarbital were 0.75 and 0.85 min, respectively, with a total chromatographic run time of 1.3 min.

### 2.4. MS conditions

The mass spectrometer was interfaced via a Sciex heated nebulizer probe with the LC system. The heated nebulizer probe temperature was maintained at 500°C and gas-phase chemical ionization was performed by a corona discharge needle at −4.85 μA using negative ion atmospheric pressure chemical ionization (APCI). The nitrogen supply for the nebulizing gas was set at a

pressure of 50 psi. The auxiliary flow and curtain gas ($N_2$) flow was set as 2.5 and 0.8 l/min, respectively. The orifice potential and electron multiplier settings were −50 and −1800 V respectively. The mass spectrometer was operated at unit mass resolution. The first quadruple filter (Q1) was programmed to select the masses of 251.1 (PHT) and 225.0 (PB). Collision induced fragmentation was performed at Q2 (collision-activated dissociation (CAD) gas thickness was set at $300 \times 10^{13}$ molecules/$cm^{-2}$). The product ions 101.9 (PHT) and 41.9 (PB) were monitored at Q3. The dwell time for each transition was 120 ms. The peak area ratio of PHT to PB obtained from selected reaction monitoring was used to construct calibration curves using weighted (1/concentration$^2$) linear regression of standard concentration versus measured peak area ratio. Plasma concentrations of unknowns were determined by interpolation from the calibration curve. Data collection and processing were performed using MacQuan PE-Sciex software.

### 2.5. Standard solutions

Two independent 5 mg/ml stock solutions of PHT were prepared by dissolving 50 mg in 10 ml methanol. Employing serial dilution, stock solutions of 1, 0.1, 0.01 and 0.001 mg PHT/ml methanol were prepared from both 5 mg/ml stock solutions. These solutions were stored at 4°C and discarded after 3 months. One set of stock solutions was used to for the preparation of calibration standards; the other set for the preparation of quality control samples. The internal standard solution of PB was also prepared as a stock solution of 1 mg/ml in methanol. A working PB solution of 200 ng/ml was prepared by serial dilution of the stock with Milli-Q water.

To prepare human plasma ultrafiltrate approximately 15 ml human plasma was decanted into a Centriprep-30® centrifugal concentrator (Amicon, MA) sample container that had a molecular weight cut-off filter of 30 000 Da. The plasma was centrifuged in this device at $1500 \times g$ maintained at approximately 37°C to harvest the resultant ultrafiltrate. Human plasma ultrafiltrate standards and quality control samples were prepared using the PHT stock solutions. For each curve, seven calibration standards were prepared distributed over the range 5–500 ng/ml. All pools of standards and quality control samples were subdivided into 0.3 ml aliquots and stored at −20°C.

### 2.6. Sample preparation

After thawing, 1 ml aliquots of plasma samples were pipetted into Centrifree ultrafiltration (Amicon, MA) devices with a molecular weight cutoff filters rated at 30 000 Da. The samples were centrifuged for 20 min at $2500 \times g$ at approximately 37°C. A 0.2 ml aliquot of the ultrafiltrate was pipetted to a $12 \times 75$ mm disposable test tube and 100 μl of the working internal standard solution was added and the mixture vortexed. The samples were extracted by adding a 1.5 ml volume of methyl-*tert*-butyl ether to each sample. After the tubes were capped, the samples were mixed for 10 min on a multiple tube vortexer and then centrifuged at $2000 \times g$ for 5 min. Following centrifugation the mixture was placed in a dry ice/isopropanol bath for approximately 3 min to freeze the aqueous layer. The organic layer was then decanted to clean $12 \times 75$ tubes and evaporated to dryness with nitrogen using a Turbovap evaporator (Zymark, MA) with a water bath temperature of approximately 40°C. The residue was resolubilized in 150 μl 10 mM ammonium buffer:acetonitrile (70:30, v/v). A 50 μl aliquot was injected onto the LC/MS/MS system.

### 2.7. Assay validation

The analytical methodology was validated according to the terms agreed during the consensus meeting on 'Analytical Methods Validation: Bioavailability, Bioequivalence and Pharmacokinetic Studies' [7]. A six run validation was performed, five between-runs and one within-run to assess precision, accuracy, specificity and stability.

### 2.8. Assay precision and accuracy

Between-run accuracy and precision were assessed by analyzing five validation samples at four concentrations (5, 50, 200 and a dilution sample

at 2000 ng PHT/ml human plasma) in five different analytical runs. These concentrations corresponded to low, medium and high with respect to the assay standard curve range. The 2000 ng/ml sample was analyzed as a 10-fold dilution. These samples were prepared independently of standard curve samples. Within-run accuracy and precision were determined by assaying five replicates of the four concentrations in a single analytical run. Accuracy was determined as the agreement between the interpolated concentration and the nominal concentration. Assay precision was calculated as the relative S.D. expressed as a percentage of the mean observed concentrations and was reported as a percent coefficient of variation.

### 2.9. Lower limit of quantitation

The lower limit of quantitation is defined as the lowest concentration of the calibration standards that quantitates the low quality control sample with acceptable precision and accuracy. To be acceptable the low quality control sample should have a between-run and within-run accuracy of $100 \pm 20\%$ and precision of less than or equal to 20%.

### 2.10. Specificity

Potential interference from endogenous compounds was investigated by the analysis of ten different sources of human plasma (five male and five female). The response at the $m/z$ and elution regions of PHT and PB were monitored. Potential matrix effects were also evaluated.

### 2.11. Hemolysis

To investigate the effect of red blood cell hemolysis on PHT protein binding, fresh blood was divided into two aliquots: one aliquot was used for comparison, the other was hemolyzed. Hemolysis of the sample was achieved by freezing the blood at $-20$°C. The blood was then processed to plasma and both aliquots were spiked with PHT at 1000 ng/ml. Ten replicates of each sample were analyzed in a single analytical run and the mean response for each aliquot was compared.

### 2.12. Freeze–thaw stability

Samples were prepared in plasma at 1000 and 2500 ng PHT/ml plasma. Each concentration was divided into two aliquots. One aliquot was processed through one freeze–thaw cycle (a freeze–thaw cycle is defined as frozen for a minimum of 12 h at $-20$°C and thawed for 2–3 h), the other was processed through three cycles. Each aliquot, in ten replicates, was processed and analyzed for free PHT against a curve prepared in ultrafiltrate. The relative recovery between one and three freeze–thaw cycles was compared

## 3. Results and discussion

### 3.1. Method development

PHT and its internal standard pentobarbital (PB) were first characterized by MS and MS/MS by flow injection analysis to ascertain their precursor ions and to select product ions for use in selected-reaction monitoring (SRM), respectively. Introduction of phenytoin to the mass spectrometer was found to give the most intense deprotonated molecular ions using negative ion atmospheric ionization. Full scan spectra for phenytoin produced a predominant peak of $(M–H)^-$ at $m/z = 251$. Collisionally induced dissociation of the $(M–H)^-$ ion produced two major fragments at $m/z$ 42 and 102. The ion at $m/z$ 42 represented $(O{=}CN)^-$ a common fragment to the parent, potential metabolites and the internal standard. The monitoring of this transition was not pursued in order to minimize the potential for crosstalk between channels when common product ions are monitored. Additionally, lower mass product ions are generally more susceptible to interferences and elevated background than higher mass ions. The $m/z$ 102 ion was optimized as the selected product ion of the precursor at $m/z$ 251 for selected-reaction monitoring mass spectrometry. Using similar procedures the precursor ion of the internal standard was determined to be the base peak $(M–H)^-$ ion at $m/z$ 225 the SRM transition of PB was determined to be $m/z$ 225 $\rightarrow$ 42.

Table 1
Between-run and within-run validation sample statistics[a]

| | Nominal concentration (ng/ml) | | | |
|---|---|---|---|---|
| | 5.00 | 50.0 | 200 | 2000 |
| *Between-run* ($n = 5$) | | | | |
| Mean | 5.19 | 51.7 | 192 | 1970 |
| S.D. | 0.771 | 6.12 | 22.3 | 218 |
| CV% | 14.8 | 11.8 | 11.6 | 11.1 |
| AR% | 104 | 103 | 96.2 | 98.5 |
| *Within-run* ($n = 5$) | | | | |
| Mean | 4.65 | 50.3 | 202 | 2050 |
| S.D. | 0.327 | 0.803 | 5.58 | 134 |
| CV% | 7.04 | 1.59 | 2.76 | 6.53 |
| AR% | 92.9 | 101 | 101 | 103 |

[a] CV%, precision expressed as coefficient of variation (S.D./mean result)$1 \times 100$; AR%, accuracy expressed as analytical recovery (mean result/nominal concentration) $\times 100$.

Initial attempts to extract PHT from human plasma samples containing PHT by solid phase extraction using reversed-phase sorbents and protein precipitation produced analytical recoveries that were poor and variable. However, liquid–liquid extraction using ethyl acetate was found to give quantitative recoveries of the analyte. Subsequently, methyl-*tert*-butyl ether (MTBE) was substituted for ethyl acetate and also yielded quantitative recoveries. MTBE has a significantly lower freezing point than ethyl acetate ($-108$ vs. $-84$°C) which permitted the use of a dry ice/2-propanol bath to freeze the aqueous layer. Decantation of the organic layer from the frozen plasma was simpler and faster than pipeting supernatant from the bilayer, hence sample throughput was improved.

LC conditions were developed to optimize for speed, peak shape and sensitivity. Flow injection analyses with mobile phases containing varying percentages of reversed phase organic solvents produced the greatest sensitivity when acetonitrile was present in percentages greater than 50%. It was also demonstrated that the addition of basic buffers such as ammonium acetate/hydroxide to the mobile phase improved sensitivity by promoting the ionized form of the analyte, presumably by increasing the gas phase concentration of proton acceptors to deprotonate the analyte and internal standard.

An Inertsil ODS-3 column ($3 \times 50$ mm) was selected for the separation as it gave consistent peak shapes without excessive tailing and provided the appropriate peak capacity for rapid analysis. A $2 \times 20$ mm guard cartridge packed with the same material was employed to protect the analytical column from highly retained sample related components. The guard column was replaced after each analytical run. In order to focus the analytes at the head of the column and optimize peak shape, the sample extracts were resolubilized in a solvent similar but with a lower percentage of organic modifier than the mobile phase.

Non-specific binding was assessed throughout the assay range by spiking aliquots of ultrafiltrate with phenytoin. A portion of the spiked ultrafiltrate was processed through the ultrafiltration device and analyzed and the results compared to a portion that was analyzed directly without ultrafiltration. The results obtained were equivalent, demonstrating no non-specific binding occurred on the ultrafiltration device or filter.

### 3.2. Validation results

#### 3.2.1. Between-run and within-run

A summary of the between-run and within-run precision and accuracy data generated for the assay validation are presented in Table 1. The limit of quantitation, determined as 5 ng/ml met the acceptance criteria with a between-run precision of 14.8% and an accuracy of 104%. For the within-run calculation, the precision for 5 ng/ml phenytoin was 7.04% and the accuracy was 92.9%. Fig. 2 illustrates a representative SRM chromatogram at the limit of quantitation standard of 5 ng PHT/ml human plasma ultrafiltrate. Based on a $S/N = 3$ the calculated limit of detection was 1 ng PHT/ml human ultrafiltrate. The data recorded for the mid and high and dilution validation samples also demonstrated acceptable precision and accuracy.

The calibration curves were linear over the range 5–500 ng PHT/ml human plasma ultrafiltrate. Correlation coefficients of the validation calibration curves were always greater that 0.995

as determined by least squares analysis. The validation run length (a run was defined as a group of standards, controls, and samples that are processed through ultrafiltration and liquid extraction and analyzed by serial LC injection and calculated from the standards in that group) was 100 analyses that demonstrated acceptable precision and accuracy.

### 3.3. Hemolysis

The effect of plasma hemolysis was investigated by comparing the mean response of hemolyzed and non-hemolyzed human plasma enriched with PHT. The mean relative recovery for hemolyzed versus non-hemolyzed samples was 106% demonstrating that acceptable data can be generated from hemolyzed samples.

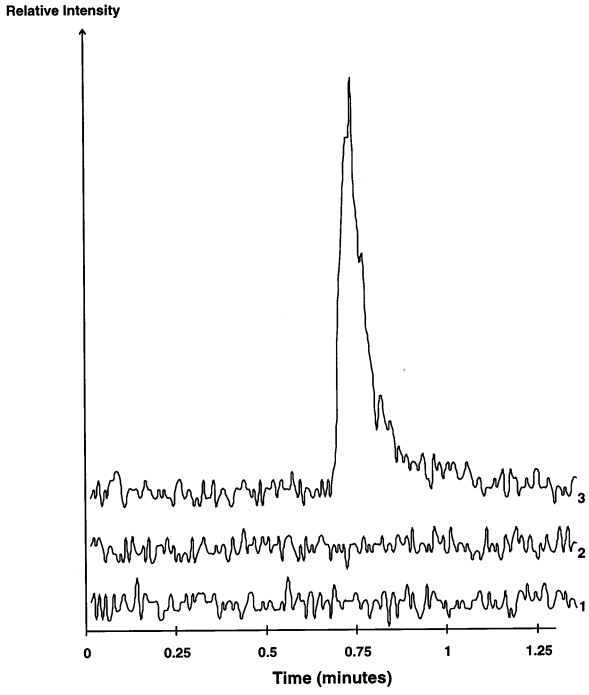


Fig. 2. Representative SRM chromatograms from the PHT ($m/z$ 251 → 102 transition) channel of extracts from (1) 200 μl blank control human plasma ultrafiltrate containing no internal standard, (2) 200 μl blank control human plasma ultrafiltrate containing internal standard, and (3) 200 μl of 5 ng PHT/ml human plasma ultrafiltrate containing no internal standard. Units: ordinate, multiplier response; abscissa: time in minutes.

### 3.4. Freeze–thaw and long term stability

PHT was shown to be stable in human plasma through three freeze–thaw cycles. The relative recoveries were determined as 101 and 93% for the 1000 and 2000 ng PHT/ml plasma, respectively.

### 3.5. Selectivity

Analysis of blank plasma ultrafiltrate from ten individuals showed no interference greater than 10% of the response of the PHT 5 ng/ml concentration. This was considered adequately selective.

As a stable isotope labeled PHT was not employed as an internal standard, experiments were conducted to assess the possibility of matrix effects. It has been identified that careful examination of matrix effects (e.g. ion suppression) should constitute an important part of assay validation for quantitative LC/MS/MS assays [8]. This was performed by comparing the response of standards prepared in the reconstitution solvent with those prepared in blank plasma ultrafiltrate after extraction. Results demonstrated no matrix effects occurred within the range of the assay.

Additionally, to support interaction studies, samples of 50 and 200 ng PHT/ml ultrafiltrate were enriched with the concomitant drug and its metabolites at clinically relevant concentrations. Selectivity was confirmed with data for precision and accuracy that was equivalent to PHT samples without the additional compounds.

The validated assay was successfully implemented for routine quantitation to support a clinical interaction study. Illustrated in Fig. 3 is an SRM chromatogram from a patient from such a study with PHT. The blood sample was collected from a subject at 90 min following a 50 mg PHT dose.

## 4. Conclusion

A simple, sensitive and rapid assay was successfully developed for the determination of free levels of phenytoin in human plasma to support clinical trials. The selectivity and sensitivity of SRM mass

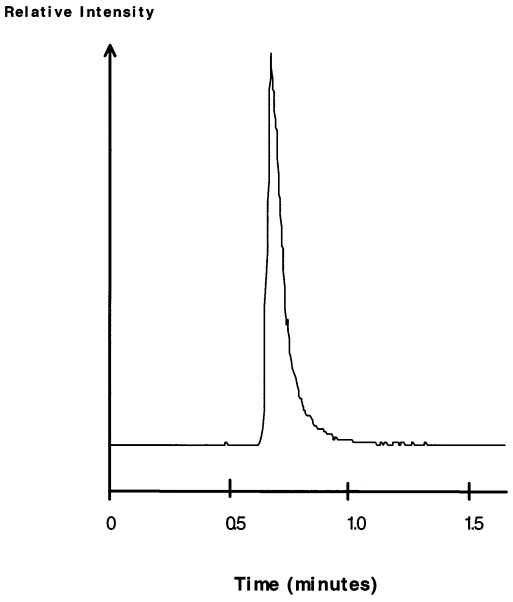


Fig. 3. SRM chromatogram from a patient sample. The blood sample was collected at 90 min following a 50 mg PHT dose.

spectrometry detection enabled a chromatographic run time of 1.5 min and a limit of quantitation of 5 ng/ml. It was also demonstrated that the developed assay can be used in the presence of concomitant medication due to the selectivity of tandem mass spectrometry. Previously reported assays for the determination of free levels of PHT suffered from lengthy sample preparation time (20–30 min per individual sample) using on-line extraction [5] or equilibrium dialysis [6]. Additionally, these assays employed UV as the detection tool which resulted in longer chromatographic run times of 6–8 min due to the selectivity of this approach and yielded higher limits of quantitation (0.25–4 μg/ml).

## References

[1] T. Kwong, Clin. Chim. Acta 151 (1985) 193–216.
[2] T.W. Rall, L.S. Schleifer, Drugs effective in the therapy of the epilepsies. In: L.S. Goodman, A.G. Gilman (Eds.), The Pharmacological Basis of Therapeutics. 7th edn., New York, MacMillan, 1988, pp. 450–454.
[3] A. Richens, Clin. Pharmacokinet. 4 (1979) 153–169.
[4] M.L. Lai, T.S. Lin, J.D. Huang, Eur. J. Clin. Pharmacol. 43 (1992) 201–203.
[5] T.D. Miller, T.C. Pinkerton, Anal. Chim. Acta 170 (1985) 295–300.
[6] K. Johansen, M. Krogh, A. Andresen, A. Christophersen, G. Lehne, K. Rasmussen, J. Chromatogr. B 669 (1995) 281–288.
[7] V.P. Shah, K.K. Midha, S. Dighe, et al., J. Pharm. Sci. 81 (1992) 309–312.
[8] B.K. Matuszewski, M.L. Constanzer, C.M. Chavez-Eng, Anal. Chem. 70 (1998) 882–889.