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Research Paper

Application of DBS Methodology for a Comparative Assessment of Phenytoin in DBS and Plasma Samples of a Pharmacokinetic Rat Study Using LC-MS/MS Method

Science

KEYWORDS : Dried Blood Spot (DBS); Bioanalysis;Pharmacokinetic (PK);LC-MS/MS; Phenytoin

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ABSTRACT

DBS is a less invasive microsampling technique which offers ethical and logistical benefits over conventional blood sampling technique. Due to dry format nature of these samples, there is also a reduced risk of pathogen infection whilehandling samples. The technique has been traditionally used as a screening tool in biomedical testing but recently its utility is emerging in drug development particularly in pharmacokinetic (PK), toxicokinetic (TK), and therapeutic drug monitoring (TDM) studies. Bioanalysis is the key element of drug development that enables accurate quantitation of drug concentrations in study samples. But DBS bioanalysis,unlike conventional plasma bioanalysis, is relatively new and quite challenging nevertheless evolving.This research work describes the application of DBS bioanalysis to measure concentrations of a therapeutic drug in rat PK samples and to draw a correlation with plasma bioanalysis. A novel and sensitive DBS-LC-MS/MS bioanalytical method for Phenytoin was developed and validated for sensitivity, precision, accuracy, stability and recovery to analyse DBS and plasma samples of a rat PK study. The observed mean PK profile of DBS and plasma demonstrated a good correlation supporting the rationale to apply theDBS method to Phenytoin and similar therapeutic drugs.

INTRODUCTION

Dry blood spot (DBS) is a blood collection technique used to collect capillary blood samples by finger or heal prick for spotting it on a filter paper or a special type of paper prior to analysis. The sample spot is dried and analysed by different analytical techniques for scientific investigations (1).The technique was first described in 1913 for the estimation of blood glucose concentration (2) and was later established as a screening tool in 1963 when Robert Guthrie and Susi reported the use of filter paper to spot blood samples from newly born babies for screening of phenylalanine amino acid to detect a rare treatable genetic disorder known as phenylketonuria (3, 4, 5).

DBS was primarily used for neonatal screening and diagnostic assays (4), DNA based assays, immunoassays and enzyme activity assays (6).In the last few years DBS has emerged as a useful microsampling tool in drug discovery and drug development for safety and efficacy evaluations (4) in animal and human studies such as pharmacokinetic (PK), toxicokinetic (TK) studies (7),therapeutic drug monitoring (TDM), clinical pharmacology, clinical pharmacogenetics (6, 8) and toxicology (9). These studies have been conducted to measure the concentrations of therapeutic drugs like cardiovascular drugs (10), antiretroviral drugs (11), antiepileptic drugs (12), immunosuppressant drugs (13, 14), antibiotics (15), psychoactive drugs (16), Vinca alkaloids (17) and drugs of abuse (18, 19) for medical investigations and to study the safety and efficacy profiles of known therapeutic drugs.

DBS is relatively a simpler alternative sampling technique with multiple advantages over conventional sampling technique. The DBS sampling typically needs less sample volume (0.1mL or less by finger or heel prick) is the major advantage over conventional sampling which typically needs more sample volume(0.5mL or more by venipuncture). Due to less invasive and microsampling nature of DBS technique, it promotes ethical use of animals in PK and TK studies (7, 20) and is user friendly for home sampling especially in patients and children during clinical investigations and TDM studies.

To estimate the drug concentrations accurately in DBS samples, sensitive and robust bioanalytical methods are required. DBS coupled with LC-MS/MS has been increasingly employed for this purpose.

From bioanalysis standpoint, the major difference between DBS and conventional plasma sample (harvested from whole blood for analysis)lies in quantitation of drug in whole blood versus in plasma. Dried blood spot samples are in essence the same as whole blood samples and have most of the same issues as those of the blood matrix concerning estimation and interpretation of pharmacokinetic parameters. Total drug measurement (sum of bound and unbound drug) from either DBS or plasma need to be considered a surrogate for the unbound plasma concentration of the drug, which is the more relevant driver than total concentration of essentially all events (pharmacokinetics and pharmacodynamics) within the body (21).

The key challenges at present to use DBS as an alternative tool in bioanalysis are related to partitioning of drug between blood and plasma, accuracy of spotting volume on DBS card and impact of hematocrit. While new tools and procedures are evolving to overcome these challenges, a point by point comparison of an emerging technique with the established technique cannot provide complete answers. Therefore, a convenient approach needs to be employed by performing a direct comparison of drug concentrations of analysed plasma and DBS samples of an in-vivo study. An acceptable correlation between concentrations maysupport the rationale touse DBS as an alternative tool in routine PK,TK and TDM studies when observed differences do not impact the overall conclusions of the study.

This research paper describes a comparisonof Phenytoin con-

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centrations from analysed DBS and plasma samples of a rat PK study for feasibility of DBS applicationin routine animal and human studies.Phenytoin is a hydantoin derivative used as anticonvulsant and it acts by blocking sodium-dependent action potentials through enhanced steady state inactivation leading to decrease sustained high frequency repetitive firing of action potentials (22, 23). Phenytoin has dose dependent long half-life and narrow therapeutic window, therefore therapeutic drug monitoring is essential to monitor clinical outcome (24). The monitoring by DBS sampling offers a better compliance in studies using such therapeutic drugs.

The Phenytoin method described in this paper is unique in terms of its methodology and sensitivity at 1.027ng/mL. The time-concentration data generated from the DBS and plasma samples of the rat PK study were comparable. USFDA in 2013 classified DBS as new bioanalytical technology in draft guidelines on method validation and encourages the development and use of new bioanalytical techniques(25). This research work provides a useful insight into successful application of DBS method to quantify a therapeutic drug in a rat study and also supports FDA thinking. This established DBS methodology can conveniently be applied to multiple studies for Phenytoin and drugs of similar nature.

EXPERIMENTAL

- **Chemicals and Reagents**

Phenytoin (Purity: 97.3%)and Carbamazepine (Purity: 99.5%)as presented in **Figure 1**were obtained from Sigma Aldrich, USA. HPLC grade Acetonitrile and Methanol were supplied by Merck, India. Formic acid was supplied by Sigma Aldrich, USA. Milli-Q water of 18.2 MΩ/cm resistivity was obtained from in-house Milli-Q water purification system of Millipore, USA. The DMS (dried matrix spotting)cards obtained from Agilent, USA, were used for sample spotting. The rat whole blood and rat plasma for blanks was obtained from in-house animal facility of Nektar Therapeutics,Hyderabad.

- **Analytical instrumentation**

API-4000 mass spectrometer of AB Sciex, USA with Electron Spray Ionization (ESI) and multiple reaction monitoring (MRM) mode was used as a detector for data acquisition. Peak integration and calibration were carried out by in-built Analyst 1.5.1 software.The ESI positive and negative ion modes were tested with different mobile phase combinations like methanol, acetonitrile and water/ammonium acetate buffer/formic acid. MS and MS/MS condition for Phenytoin (analyte) and carbamazepine (internal standard, ISTD) were optimized by continuous infusion using syringe pump at5 µL/min.

A Shimadzu SIL HTc – 20 AD (Shimadzu Corporation, Japan) consisting of flow control valve, column oven, degasser and auto sample operated in gradient mode to deliver the mobile phase. The chromatographic system consisted of reverse phase C_{18} column, Intrada-WP RP 150 mm ×3 mm, 3µ internal diameter (Imtakt, USA).

Two different mobile phases, mobile phase A and mobile phase B with gradient flow were used during the analysis. The composition of mobile phase A was water and acetonitrile (95:5, v/v) with 0.1% formic acid.The composition of mobile phase B waswater and acetonitrile (5:95, v/v) with 0.1% formic acid.Gradient flow with 0.7 mL/minute was used during analysis for total run time of 6.0 minutes. The auto sampler and column temperature were set at 5°C and 50°C respectively and the 20 µL injection volume was used during analysis.

- **Preparation of stock, calibration standards and quality control samples**

1.0 mg/mLof Phenytoin and Carbamazepine stock solutions was prepared in methanol: water (50:50, v/v) mixture and stored at -20°C. 500 ng/mL of carbamazepine was prepared in methanol: water (50:50, v/v) mixture and used as a working internal standard (WISTD). The working solution of (calibration standards and quality control samples)Phenytoin were prepared in methanol: water (50:50, v/v)mixture.The calibration curve (CC) (range:1.027to 1027.488 ng/mL) and quality controls (QCs) (2.452, 98.078, 490.392, 817.320ng/mL as Low QC, Low QC-II, Mid QC and High QC respectively) were prepared by spiking working solution in blank Sprague Dawley (SD) rat blood and rat plasma.

- **Sample preparation**

The sample preparation conditions were optimized during method development. These included selection of DBS card, spotting of sample on DBS card, selection of extraction solvent and volume of extraction, sample drying time and sample punching for reproducibility and optimum extraction efficiency. Ethyl acetate was used as solvent for extraction of analyte and ISTD from plasma and DBS samples.

Fresh SD rat blood was collected in K2EDTA coated tubes. 5µl of analyte working solution was spiked into a sample tube containing 95 µl of whole blood and mixed gently. 15 µl of mixed sample was spotted on the Agilent DMS card. The spotted card was allowed to dry overnight at room temperature in a vacuum desiccator containing activated silica gel.

The whole dried spots were cut neatly and transferred into a centrifuge tube. 1.0 ml of extraction solvent (ethyl acetate), 100µl of 0.2% ammonia and 10µl of ISTD (500ng/mL of carbamazepine) were added to the sample tube. The contents was mixed for 10 minutes and centrifuged at 14000 rpm for 10 minutes. The supernatant was collected and evaporated under gentle stream of nitrogen in an evaporator. The dried extract samples were reconstituted with 150 µL of reconstitution solution (0.2% Formic acid in Water: Methanol,(20:80, v/v). 20µLreconstituted sample was injected into chromatographic system.

- **Method validationand application to rat PK study**

The method parameters evaluated were:linearity, extraction efficiency(recovery), precision and accuracy and bench top stability in DBS. The set acceptance criteria for accuracy (% bias) of CC & QC samples were ± 15% of their respective nominal concentrations except at Limit of quantitation (LOQ), where it wasset ± 20% in accordance with the current regulatory guidelines (25, 26).The in-study method performance was demonstrated by successful application of this method in rat PK study.

o ***LOQ determination***

The LOQ was determined based on analyte response by spotting six samples each with LOQ concentration of analyte and six blank samples on DBS cards. The LOQ response was compared with blank samples. The acceptance criteria for interference peak area at analyte retention time (RT)was ≤ 20% of the peak area of LOQ and for interference peak area at the ISTD RT was ≤ 5%.

o ***Linearity***

Three precision and accuracy (P&A) batches were analyzed to compare the intra-assay and inter-assay on three different days. The standard calibration curves were constructed using the peak area ratio of Phenytoin and carbamazepine with Phenytoin nominal concentrations in whole blood spotted on DBS (calibration standards concentrations at1.027, 2.055, 5.137, 20.550, 51.374, 102.749, 205.498, 513.744, 821.990, 1027.488ng/mL). Quadratic regression with weighting factor $1/X^2$ was performed to assess the linearity. In addition, a DBS blank (whole blood spotted onDBS) and a DBS zero blank sample (whole blood spotted on DBS and ISTD added during sample preparation) were run to

demonstrate the absence of interferences at analyte and ISTD retention time.

o ***Extraction efficiency (Recovery)***

The recovery experiment was performed at four QC levels (Low QC, Low QC-II, Mid QC and High QC at 2.452, 98.078, 490.392, 817.320ng/mLrespectively) with four replicates at each concentration level. The mean area of extracted samples compared with unextracted (Aqueous sample) samples at each respective QC levels to evaluate the extraction efficiency.

o ***Precision and Accuracy***

Precision and accuracy of the method was evaluated using three different batches of quality control samples at five different concentrationsof1.027, 2.452, 98.078, 490.392 and 817.320ng/mL. Each batch was quantified with a specific calibration curve. Four replicates of quality control samples at five concentration levels were used to evaluate the intra-batch precision (%CV) and accuracy (% nominal). The inter-batch assay precision and accuracy were determined from three analyzed batches to obtain the corresponding inter-batch precision and accuracy.

o ***Stability***

Freshly spiked 389.200 ng/mL samples were spotted on DBS cards and secured on bench top at ambient temperature for stability assessment. After 11 days,the DBS stability samples were processed and analyzed along with freshly spiked and spotted comparison QC and CC Samples.

o ***Partial validation in rat plasma***

Partial validation of Phenytoin in plasma was performed in SDrat plasma by spiking QCs in plasma for analyzing inter and intra precision and accuracy batches to support analysis of plasma samples of rat PK study.

• **Application of Phenytoinmethods for analysis of DBS and plasma samples from rat PK study**

The rat study coded as LS-2012-921 was conducted for pharmacokinetic evaluation of Phenytoin following intravenous (IV) administration of Phenytoin in rats to compare bioanalytical data from plasma and dry blood spot (DBS) samples. Whole blood samples were collected through carotid artery cannula at respective time points (0.03, 0.08, 0.25, 0.5, 1.0, 2.0 and 4.0 hours) from rats (N=4) and immediately transferred to K2EDTA microtainer tube. Samples, placed in ice bath, were mixed by inversion. From each tube 15µL of blood sample was spotted on Agilent DMS cards in duplicate and the sample card was allowedto dry at ambient temperature. From remaining whole blood samples, plasma was harvested by centrifugation at 10,000 RPM for 5 minutes. Plasma samples was transferred into micronics tubes in duplicate and stored at -70°C until analysis. The DBS and plasma samples were analyzed by above mentioned method. The procedure adopted during sample preparation and analysis of DBS and plasma samples were identical except for sample collection procedure.

RESULTS AND DISCUSSION

• ***Mass spectrometryand Chromatography***

Positive ion mode was selected for detection and quantitation of analyte, which on fragmentation produced prominent and stable product ions. The protonated molecular ion $[M + H]^+$ of the analyte and ISTD were observed at 253.0 and 237.1Da respectively. The m/z of 253.0/182.0 and 253.0/104.0 transition for analyte and 237.1/194.0 transitions for ISTD were monitored.The analysis was carried out in ESI positive ion mode with spray voltage set at 5500 V. The mass spectrometer parameters are presented in **Table 1.**

The chromatography obtained with optimized gradient mobile phase of acidified acetonitrile and water was acceptable in terms of peak width and peak shape. Optimum results were achieved with reverse phase C_{18} column (Intrada-WP RP 150mm× 3mm, 3µ)with total analysis run time of 6.0 min. The retention time for analyte and ISTD in DBS samples was around 3.0 minutes and in plasma samples it was around 3.7 minutes. The optimized chromatographic conditions are presented in **Table 2**.Representative chromatograms of extractedblank from DBS and plasma are presented in **Figure 2 and 3**.Representative chromatograms of LOQ from DBS and plasma samples are presented in **Figure 4and5**.

• ***LOQ determination***

The criterion to define the Limit of quantitation (LOQ) of Phenytoin was based on comparison between noise level or peak area of the analysed blank samples and the peak response of Phenytoin in the spiked samples at analyte retention time. LOQ response was more than five times of noise level in the blank samples. The precision and accuracy of the analyte at 1.027 ng/mL (LOQ)was < 20%. The method precisely quantified LOQ at 1.027 ng/mL in 15 µL DBS blood spot. Sensitivity data is presented in **Table3.**

• ***Linearity***

The peak area ratio of analyte to ISTD was linear over the concentration range of 1.027 to 1027.488 ng/mLin SD rat dry blood spot. The calibration model was selected on basis of analysis of the data by different regression models. The best fit for the calibration curve was achieved by quadratic regression with $1/X^2$weighting factor. The correlation coefficients (r^2) were greater than 0.995. Representative standard curve from DBS is presented in **Figure 6**.

• ***Precision and accuracy***

The intra-day precision and accuracy for analyte wasevaluated by analysing four replicates at five quality control(QC) levels spiked on DBS card. Inter-day assays precision was determined by analyzing five QC levels in three different runs. The overall precision (% CV) and accuracy (% nominal) was within ± 15% and 85.0% to 115.0%respectively. The representative data of intra assay precision and accuracy and inter-assay precision and accuracy of Phenytoin in DBS is presented in **Table 4**.

• ***Extraction efficiency(Recovery)***

Consistent recovery was obtained with Agilent DMS card in 1 mL of ethyl acetate as extraction solvent. The matrix factor was within acceptable limits did not contribute to ion suppression or ion enhancement. The extraction efficiency was calculated betweenpeak areas of extracted sample and aqueous sample. Recovery (%) of analyte at Low QC, Low QC-II, Mid QC and High QC was 56.4, 58.6, 53.4 and 56.7 respectively. The mean recovery of quality control samples was 56.3% with 3.8 % CV. The recovery data is presented in **Table 5**.

• ***Bench top Stability***

The Phenytoin DBS samples werestable for 11 days at ambient temperature. The percentage change between freshly spotted DBS samples and stability DBS samples was 7.7%. The stability data is presented in **Table 6**.

• ***Partial validation in plasma***

The intra-day precision and accuracy for analyte was evaluated by analysing four replicates at five QC levels. Inter-day assays precision was determined by analysing five QC levels in two different runs. The overall precision (% CV) and accuracy (% nominal) was within ± 15% and 85.0% to 115.0% respectively. The representative data of intra assay precision and accuracy and inter-assay precision and accuracy of Phenytoin in plasma is presented **Table 7**.Representative standard curve from plasma is presented in **Figure 7**.

• ***Rat PK study data comparison(By DBS and Plasma analysis)***

The Phenytoin concentrations were quantified in both DBS and plasma samplesof rat PK study LS-2012-921 by above mentioned DBS and plasma methods.The in-study method performance of QCs was demonstrated in DBS and plasma sample analysis is presented **in Table 8 and 9**. The percentage difference of mean pharmacokinetic parameters between DBS and plasma was between -19 to 21%. The sample size of the study at each time point wasN=4. The precision (%CV) of analysed samples calculated for all time points was 4.9 for plasma samples and 33.3 for DBS samples. The increased sample size in routine PK and TK studies is expected to reduce the variability in DBS. The PK parameters are presented **in Table 10**.The observed mean pharmacokinetic profile between DBS and plasma was found comparable is presented in **Figure 8**.The comparative data demonstrated acceptable correlation between phenytoin concentrations in plasma and DBS when Altman Bland agreement test was applied is presented in **Figure9** indicates applicability of this DBS and plasma methods are comparable.

CONCLUSIONS

Although DBS is an advantageous sampling technique emergingas a bioanalytical application in drug development,may not fully replace the conventional plasma bioanalytical methodologyuntil more and more data on DBS becomes available to answer key questions on current challenges to narrow down the real differences between the two techniques. However, one approach to address these challenges is to conduct a direct comparison of drug quantitation by DBS and plasma methods to establish a correlation. It will be convenient to employ DBS as an alternative approach in bioanalysis of PK, TK and therapeutic drug monitoring samples where correlations between DBS and plasma data are acceptable.The Phenytoin method at LOQ of 1.027 ng/mL by DBS-LCMS/MS described here is not previously reported. The method performance was validated by analyzing rat DBS samples. The time-concentration profile of DBS and plasma samples demonstrated an acceptable agreement between data using Altman-Bland comparison test. Based on the performance, the method is unique in terms of methodology and sensitivity and can be applied for bioanalysis of wide range of DBS samples from PK, TK and therapeutic drug monitoring studies.The approach adopted in this research work also supports USFDA thinking on exploring new technologies as mentioned in their current draft guidelines.

ETHICAL CONDUCT OF RESEARCH

The authors state that they have obtained appropriate institutional review board approval or have followed the principles outlined in the Declaration of Helsinki for all animal experimental investigations.

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Table 1: Mass spectrometer parameters of API 4000

Mode of operation			Electron Spray Ionization (positive ion mode)			
Collision activated dissociation (CAD)			10			
Curtain Gas (CUR)			25			
Ion source gas 1 (Gas 1)			40			
Ion source gas 2 (Gas 2)			50			
Ion spray Voltage (IS)			5500			
Temperature (^{o}C)			450			
Pause time (ms)			5			
Collision gas			Nitrogen			
Compound Name	Ions Monitored	Dwell time (ms)	Declustering potential (DP)	Collision energy (CE)	Collision cell exit potential (CXP)	Entrance potential (CXP)
Phenytoin	253.0 → 182.0 &104.0	100	51	49	6	10
Carbamazepine	237.1 → 194.0	100	58	29	13	10

Table 2: Chromatographic conditions

Analytical column	Intrada-WP RP, $C_{18,}$ 150 × 3 mm, 3µ			
Column oven temperature (^{o}C)	50			
Mobile phase A	0.1% formic acid in water and acetonitrile (95:5, v/v)			
Mobile phase B	0.1% formic acid in water and acetonitrile (5:95, v/v)			
Rinsing solution	0.1% formic acid in water and acetonitrile (50:50, v/v)			
Run time (min)	6			
Injection volume (µL)	20			
Time	Mobile phase A (%)	Mobile phase B (%)	Flow rate (mL/min)	
0.01	98	2	0.01	
0.50	98	2	0.50	
1.00	55	45	1.00	
2.00	20	80	2.00	

4.00	20	80	4.00	
4.50	98	2	4.50	
5.00	98	2	5.00	
6.00	Stop			

Table 3: Sensitivity of Phenytoin by using DBS and LC-MS/MS method

S. No.	Peak Area	LLOQ (1.027 ng/mL)	
		Conc. (ng/mL)	% Accuracy
1	2175	0.693*	67.5*
2	2446	0.872	84.9
3	2573	0.951	92.6
4	2411	0.873	85.0
Mean	-	0.8987	-
SD		0.04532	
% CV		5.0	
% Nominal		87.5	

*not meeting the acceptance criteria

Table 4: Intra and inter assay precision and accuracy of quality control samples for Phenytoin in DBS

Quality control concentration	LLOQ (1.027 ng/mL)	LQC (2.452 ng/mL)	LQC-II (98.078 ng/mL)	MQC (490.392 ng/mL)	HQC (817.320 ng/mL)
Intra-assay					
Mean	1.0820	2.2975	94.4550	502.2653	831.8617
SD	0.05374	0.40517	2.09986	27.19681	29.50573
% CV	5.0	17.6	2.2	5.4	3.5
% Nominal	105.4	93.7	96.3	102.4	101.8
N	4	4	4	4	4
Inter-assay					
Mean	1.0629	2.5203	98.6998	481.0931	747.5124
SD	0.19156	0.35991	5.31303	29.78792	92.80683
% CV	18.0	14.3	5.4	6.2	12.4
% Nominal	103.5	102.8	100.6	98.1	91.5
N	12	12	12	12	12

Table 5: Extraction recovery of Phenytoin by using DBS

Quality Control concentration and Peak area	LQC (2.452 ng/mL)		LQC-II (98.078 ng/mL)		MQC (490.392 ng/mL)		HQC (817.320 ng/mL)	
	Extracted Area	Aqueous Area	Extracted Area	Aqueous Area	Extracted Area	Aqueous Area	Extracted Area	Aqueous Area
Mean Area (N=4)	5564	9873	204357	348989	986633	1847106	1675078	2954837
Recovery	56.4		58.6		53.4		56.7	
Mean Recovery	56.3							
SD	2.2							
%CV	3.8							

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Table 6: Bench top stability of Phenytoin at ambient temperature (11 days post spotting)

Quality Control concentration	389.200 ng/mL	
	Comparison	Stability
Mean (N=4)	372.8473	401.5865
SD	58.27063	31.16387
% CV	15.6	7.8
% Nominal	95.8	103.2
% Change	7.7	

Table 7: Intra and inter assay precision and accuracy of quality control samples for Phenytoin in plasma

Quality control concentration	LLOQ (1.027 ng/mL)	LQC (2.452 ng/mL)	LQC-II (98.078 ng/mL)	MQC (490.392 ng/mL)	HQC (817.320 ng/mL)
Intra-assay					
Mean	1.0660	2.4858	97.3610	512.3180	766.5143
SD	0.11922	0.40496	2.97752	17.13795	22.01360
% CV	11.2	16.3	3.1	3.3	2.9
% Nominal	103.8	101.4	99.3	104.5	93.8
N	4	4	4	4	4
Inter-assay					
Mean	1.0209	2.5601	97.4951	498.5049	761.9141
SD	0.14324	0.30293	3.10954	19.66665	29.72370
% CV	14.0	11.8	3.2	3.9	3.9
% Nominal	99.4	104.4	99.4	101.7	93.2
N	8	8	8	8	8

Table 8: Summary of quality control data of Phenytoin in DBS during study sample analysis
(In-study method performance in DBS samples of study: LS-2012-921)

S. No.	LQC (2.438 ng/mL)		LQC-II (97.521 ng/mL)		MQC (487.606 ng/mL)		HQC (812.676 ng/mL)	
	Conc. (ng/mL)	% Accuracy	Conc. (ng/mL)	% Accuracy	Conc. (ng/mL)	% Accuracy	Conc. (ng/mL)	% Accuracy
1	3.136	128.6	98.474	101.0	544.511	111.7	806.811	99.3
2	3.824	156.8	99.632	102.2	549.154	112.6	760.379	93.6

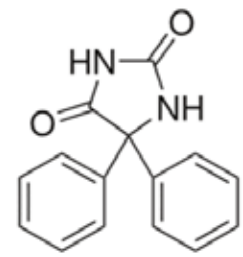
Table 9: Summary of quality control data of Phenytoin in plasma during study sample analysis
(In-study method performance in plasma samples of study: LS-2012-921)

S. No.	LQC (2.438 ng/mL)		LQC-II (97.521 ng/mL)		MQC (487.606 ng/mL)		HQC (812.676 ng/mL)	
	Conc. (ng/mL)	% Accuracy	Conc. (ng/mL)	% Accuracy	Conc. (ng/mL)	% Accuracy	Conc. (ng/mL)	% Accuracy
1	1.967	80.7	88.727	91.0	420.763	86.3	719.237	88.5
2	2.459	100.9	83.023	85.1	487.571	100.0	759.064	93.4

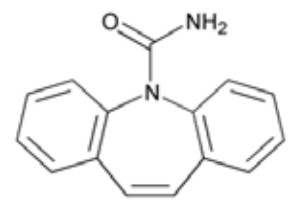
Table 10: Comparison of mean pharmacokinetic parameters of Phenytoin (DBS vs plasma)

PK Parameter	Units	Plasma	DBS	% Difference
C0	(ng/mL)	1515	1493	1
AUCINF_obs	(hr*ng/mL)	274	229	17
Vss	mL/kg	5513	4357	21
Cl_obs	(mL/hr/kg)	7483	8918	-19
Cmax	(ng/mL)	943	823	13

Figure 1- Chemical structure of Phenytoin and Carbamazepine



Phenytoin (Analyte)



Carbamazepine (Internal Standard)

Figure 2: Representative Chromatogram of an extracted blank from DBS

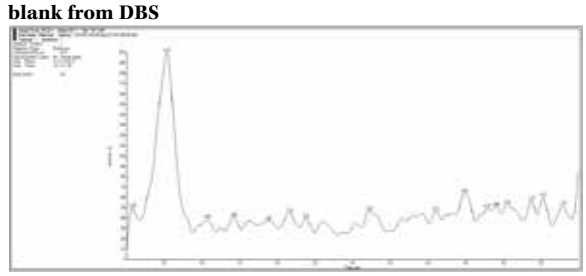


Figure 3: Representative Chromatogram of an extracted blank from Plasma

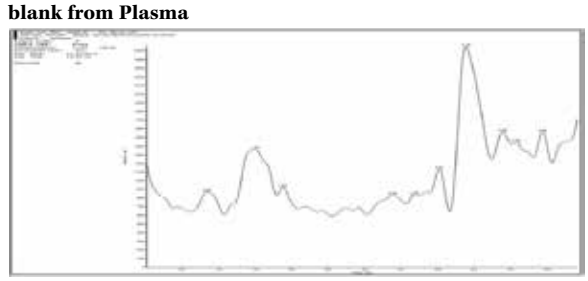


Figure 4: Representative chromatogram of Phenytoin LOQ and Internal Standard from DBS card

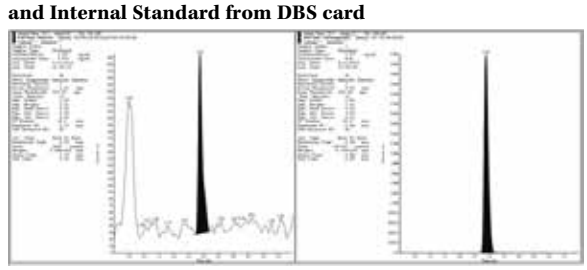


Figure 5: Representative chromatogram of Phenytoin LOQ and Internal Standard from Plasma

Figure 6: Representative calibration curve of Phenytoin from DBS

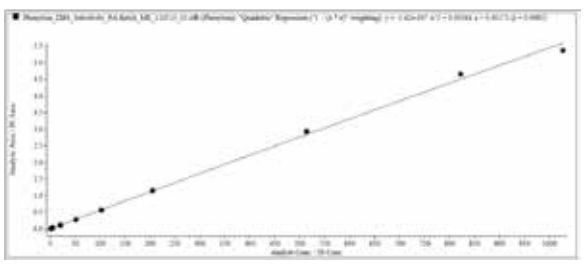


Figure 7: Representative calibration curve of Phenytoin from Plasma

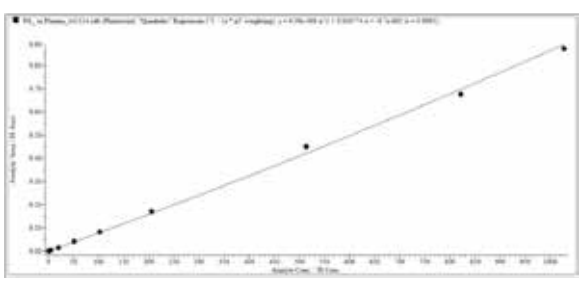


Figure 8: Mean PK profile of Phenytoin following IV administration (DBS vs plasma; N=4)

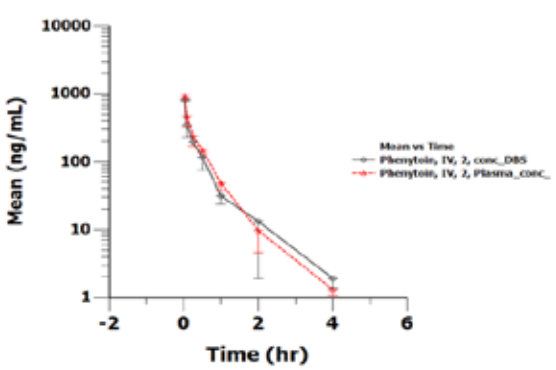
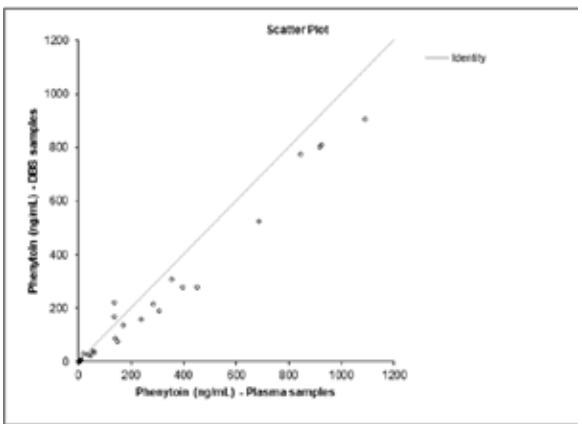
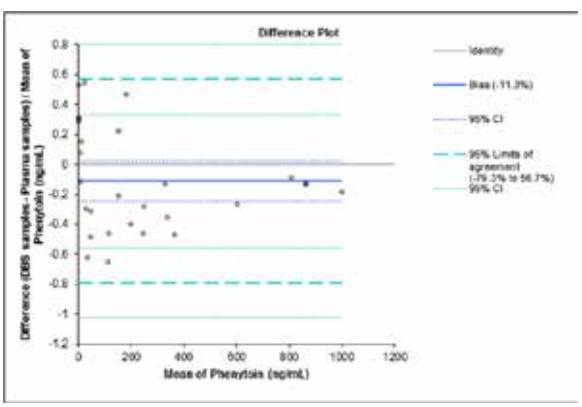


Figure 9: Scatter and difference Plot(Altman-Bland agreement test)





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