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Validation of HPLC-MS Method in Positive Ion Mode for Estimation of Phenytoin in Human Plasma Using Phenytoin D10 as Internal Standard

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A high performance liquid chromatography mass spectrometric method for the estimation of phenytoin, in human plasma in positive ion mode was developed and validated using phenytoin D10 as internal standard (IS). Sample preparation was accomplished by solid-phase extraction technique. The eluted samples were chromatographed on Zorbax Eclipse XDB-Phenyl 4.6 × 75 mm, 3.5 µm (Make: Agilent Technologies) column using a mobile phase consisting of HPLC grade acetonitrile: 0.1 % formic acid (80:20 v/v). The method was validated over a concentration range of 101.253 to 10074. 937 ng mL^{-1} for phenytoin. This validation report provides the results of selectivity, matrix effect, sensitivity determinations, precision and accuracy data, recovery and dilution integrity along with all pertinent documentation. An open label, balanced, randomized, two-treatment, two-period, two-sequence, cross-over, single oral dose bioequivalence study of phenytoin sodium 100 mg capsules (3 × 100 mg, a total of 300 mg) of Green Evolution Laboratories, India comparing with phenytoin sodium flynn 100 mg hard capsules (3 × 100 mg, a total of 300 mg) of Flynn Pharma Ltd, Alton House, Ireland in healthy, human adult subjects, under fasting conditions was conducted. The 90 % confidence intervals were calculated for the C_{max}, $AUC_{(0-t)}$ and $AUC_{(0-\infty)}$, giving values between 96.26-110.38 % demonstrating the bioequivalence of the two formulations.

Keywords: Phenytoin, Phenytoin-D10, Human plasma, LCMS/MS, Pharmacokinetic study.

INTRODUCTION

Epilepsy a most common central nervous system disorder may cause persistent deformity and decreasing the quality of life, affecting 50 million people worldwide [1]. Epilepsy is a chronic disorder that can influence human physiological, social and vocational functioning. The rate of mood disorders is higher in patients with epilepsy than in those with other chronic medical conditions, like diabetes or asthma [2-4]. Phenytoin is chemically known as 5,5-diphenyl-imidazoline-2,4-dione (Fig. 1) is one of the most widely used anticonvulsant drugs for the treatment of many epileptic-type seizures and is usually given orally in doses ranging from 200 to 600 mg per day [5-7]. It is highly bound to the carrier protein human serum albumin and is mostly excreted in bile as inactive metabolites, which are then reabsorbed from the intestinal tract and excreted in the urine [6,8]. Poor water solubility, non-linear dose-dependent pharmacokinetics and a narrow therapeutic window are the three pharmacologic characteristics associated with the risk of non-bioequivalence after the use phenytoin as generic drug [7-10]. The reported analytical methods used to determine phenytoin concentrations in biological samples includes biointeraction studies based on high performance affinity chromatography to investigate the binding of human serum albumin (HSA) to two major phenytoin metabolites [6] comparative bioavailability of a single oral 200 mg dose of four brands of phenytoin sodium available in the Indian market [7], to determine the effect of gender and menstrual cycle on phenytoin bioavailability of 100 mg extended phenytoin sodium capsules using HPLC assay [9] binding characteristics of phenytoin to serum proteins in the Japanese population [8] generic substitutions for antiepileptic drugs [10], determination of free and total levels of phenytoin in human plasma from patients with epilepsy by MEKC [11] (micellar electro kinetic chromatography), pharmacokinetic profile of a newly developed 300 mg extended phenytoin sodium capsule (dilantin) formulation [12] double blind randomized study comparing brand name and generic phenytoin [13] comparative study of the bioavailability and dissolution of seven formulations of phenytoin [14], HPLC determination of lamotrigine in serum with phenytoin, carbamazepine and carbamazepine epoxide [15], simultaneous determination of phenytoin, carbamazepine

and 10,11-carbamazepine epoxide in human plasma by high-performance liquid chromatography with ultraviolet detection [16], Simultaneous determination of lamotrigine, phenobarbitone, carbamazepine and phenytoin in human serum by high-performance liquid chromatography [17], quantification of carbamazepine, carbamazepine-10,11-epoxide, phenytoin and phenobarbital in plasma samples by stir bar-sorptive extraction and liquid chromatography [18], simultaneous determination of seven antiepileptic drugs (AEDs), including primidone, phenobarbital, phenytoin, carbamazepine with its two major metabolites carbamazepine-10,11-epoxide and carbamazepine-10,11-(*trans*)-dihydrodiol in serum by high performance liquid chromatography (HPLC)-diode array detector (DAD) [19], HPLC assay method for the simultaneous determination of phenytoin and fosphenytoin in human plasma and plasma ultrafiltrate [20] and liquid chromatography-tandem mass spectrometry (LC-MS/MS) [21,22].

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 [7], ultra filtration followed by HPLC with UV detection using liquid-liquid extraction with the long run time of 17 min [8] and automated sequential trace enrichment of dilalysate (ASTED) with HPLC with UV detection [10]. Some others RP-LC methods were reported for the determination of phenytoin in pharmacokinetics and bioequivalence studies, but without detailed description of the method and the validation parameters [7,12-14]. Also, LC methods for the simultaneous determination of phenytoin and other anticonvulsant drugs in biological matrix were developed using solid-phase extraction disk to separate the drugs [15], protein precipitation extraction [16,17], stir adsorptive extraction [18] and solid-phase extraction using disposable cartridges with 1 mL sample volume capacity [19]. Moreover, the RP-LC method with UV detection was developed in the linear range from 0.4-400 μg mL^{-1} for the simultaneous determination of phenytoin and its prodrug fosphenytoin in human plasma and plasma ultrafiltrate [20], HPLC method for the determination of phenytoin in plasma or whole blood [21] LCMS assay for the determination of free levels of the highly protein bound drug phenytoin (5,5-diphenylhydantoin) in human plasma by the drug/protein complex by ultrafiltration [21] and positive electrospray ionization [22-27], with liquid-liquid and protein precipitation extraction are reported, respectively. Determination of phenytoin in human plasma by gas chromatography is reported [28]. These approaches have associated with disadvantages including long sample preparation time and analysis time and high limits of quantitation and lack of complete validation procedures. To overcome the disadvantages of the above mentioned methods, we have studied a fully validated time high performance liquid chromatography mass spectrometric method for the estimation of phenytoin, in human plasma using Phenytoin D10 as an internal standard (IS). The usage of deuterated internal standard gives accurate results. The present method employs a simple solid-phase extraction (SPE) technique for sample preparation and phenytoin-d10 was used for the first time as an internal standard for the high performance liquid chromatography mass spectrometric method used for the estimation of phenytoin in human plasma in positive ion mode. The present method has a better runtime, recovery, precision and accuracy, matrix effect than that of the all previously reported methods. An open label, balanced, randomized, two-treatment, two-period, two-sequence, cross-over, single oral dose bioequivalence study of phenytoin sodium 100 mg capsules (3 × 100 mg, a total of 300 mg) of Green Evolution Laboratories, India comparing with phenytoin sodium flynn 100 mg hard capsules (3 × 100 mg, a total of 300 mg) of Flynn Pharma Ltd, Alton House, 4 Herbert Street, Dublin 2, Ireland in healthy, human adult subjects, under fasting conditions was conducted. The 90 % confidence intervals were calculated for the C_{max}, $AUC_{(0-t)}$ and $AUC_{(0-\infty)}$, giving values between 96.26-110.38 % demonstrating the bioequivalence of the two formulations. The chemical structures of phenytoin and phenytoin-d10 (internal standard) are shown in Fig. 1.

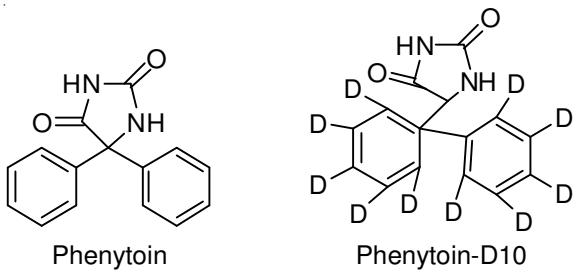

Fig. 1. Chemical structures of phenytoin and phenytoin-d10 (internal standard)

EXPERIMENTAL

Phenytoin *N*,*N*-dimethyl-2-(6-methyl-2-*p*-tolylimidazo-[1,2-a]pyridin-3-yl)acetamide, hemitartrate) Purity: 100.0 % (as is basis) obtained as a gifted sample from Splendid Labs Hyderabad, India. Phenytoin D10 purity: 99.16 % (as is basis) obtained as a gifted sample from Splendid Labs, Hyderabad, India. HPLC Grade acetonitrile purchased from J.T. Baker (Philipsburg, USA). Analytical grade formic acid was purchased from Merck, Zorbax Eclipse XDB-Phenyl 4.6 × 75 mm 3.5 μm (Make: Agilent Technologies) and HPLC grade water purchased from rankem. Water used in the entire analysis was prepared by a milli-Q water purification system (Millipore, Bangalore, India). The human K2EDTA control plasma was obtained from Vuppala Venkaiah memorial blood bank, (Hyderabad, India).

Chromatographic separation was carried out using a HPLC (LC 2010C, Shimadzu Corporation, Kyoto, Japan) equipped with a quaternary pump, a degasser, an autosampler, an injector with a 100 μL loop, a column oven, a UV detector and a data system (Class VP version 6.12). The analytical column used was Zorbax Eclipse XDB-Phenyl 4.6 × 75 mm, 3.5 μm for the desired separation. The mobile phase consisted of HPLC grade acetonitrile: 0.1 % formic acid (80:20, v/v). The flow-rate of the mobile phase under isocratic condition was kept at 1.0 mL min^{-1}, injection volume was 15 μL. The sample cooler temperature was maintained at 15 °C and the total run time was about 2.0 min. Mass spectrometric detection was carried out using an API 3000 tandem mass spectrometer (Applied Biosystems/MDS SCIEX, Toronto, ON, Canada)

equipped with an electrospray ionization (ESI) source. The mass spectrometer was operated in positive ion mode and the tandem mass spectrometry conditions for phenytoin and the internal standard (IS, Phenytoin-D10) were optimized by carrying out full scans in positive ion detection mode.

Preparation of stock and working solutions

Phenytoin stock solution: 5.0124 mg of phenytoin working standard was transferred to a 5 mL clean glass volumetric flask, dissolved in HPLC grade methanol and made up to the volume produce a solution of 1 mg mL^{-1}. Corrected the above concentration of phenytoin solution accounting for its potency and the actual amount weighed. The stock solution was stored in refrigerator at 2-8 °C and used for maximum of 166 h. The stock solutions were diluted to suitable concentrations using a mixture of acetonitrile and HPLC grade water in the ratio of (60:40 v/v) for spiking into plasma to obtain calibration curve (CC) standards, quality control (QC) samples and DIQC samples. For the preparation of calibration curve standards and quality control samples separate stock solutions were used. All other final dilutions (system suitability dilutions, aqueous mixture, *etc.*) were prepared in mobile phase.

Phenytoin D10 stock solution (internal standard): Weighed about 5.1642 mg of phenytoin D10 transferred to a 5 mL volumetric flask, dissolved in HPLC grade methanol and made up the volume with the same to produce a solution of 1 mg mL^{-1}. Corrected the above concentration of phenytoin D10 accounting for its molecular weight, potency and the actual amount weighed. The stock solution was stored in refrigerator at 2-8 °C and used for maximum of 166 h.

Calibration curve standards and quality control samples: Calibration curve standard consisting of a set of ten non-zero concentrations ranging from 101.253 to 10074.937 ng mL^{-1} of phenytoin were prepared. Prepared quality control samples consisted of concentrations of 101.437 ng mL^{-1} (LLOQ QC), 301.897 ng mL^{-1} (LQC), 1509.484 ng mL^{-1} (MQC1), 5031.615 ng mL^{-1} (MQC2) and 7549.310 ng mL^{-1} (HQC) for phenytoin. These samples were stored at –70 °C until use. Twelve sets of LQC and HQC were stored at –20 °C deep freezer to check –20 °C stability. Twelve sets of quality control samples for dilution integrity were prepared by spiking about 1.60 times (16139.903 ng mL^{-1}) and another twelve sets by spiking about 3.2 times (32279.807 ng mL^{-1}) highest standard concentration of phenytoin. From these, six sets each of two times dilution and four-time dilution was performed.

Sample preparation: The samples were thawed at room temperature and vortexed to ensure complete mixing of the contents. 250 µL of the plasma sample was pipetted in toc pre-labelled RIA vial tubes. 25 µL of internal standard dilution (phenytoin D10 at a concentration of 50 ng mL^{-1}) was added to it and vortexed, except in blank plasma samples where 25 µL diluent was added and vortexed. Then 250 µL of 0.1 % formic acid was added and vortexed. The sample mixture was loaded onto Strata-X 33 µm polymeric sorbent cartridge (30 mg mL^{-1}) that were pre-conditioned with 1.0 mL of HPLC grade methanol followed by 1 mL Milli Q water (new cartridge for each sample). After applying the maximum pressure the extraction cartridge was washed with 2 mL of Milli Q/HPLC grade water (1 mL of each time). Then the samples were eluted with 2 mL of eluent, *tert*-butyl methyl ether and evaporated up to dryness at 45 °C under gentle stream of nitrogen. The dried extract sample was reconstituted with 500 µL of the mobile phase and transferred into auto sampler loading vials and loaded in the auto sampler and injected.

Method validation: Thorough and complete method validation of phenytoin in human plasma was carried out for selectivity, sensitivity, linearity, precision and accuracy, recovery, matrix effect, dilution integrity and stability according to US Food and Drug Administration (FDA, 2001 and European Medicines Agency (EMEA) 2009 [29,30].

Selectivity: Selectivity is the ability of an analytical method to differentiate and quantify the analyte in the presence of other components in the sample. The selectivity of the method was evaluated by processing six different lots of blank plasma sample. These samples were spiked with LLOQ concentration along with internal standard to confirm the lack of interference at their retention time and absence of lot to lot variation.

Linearity: A regression equation with a weighting factor of $1/(\text{concentration ratio})^2$ of drug to internal standard concentration was judged to produce the best fit for the concentration-detector response relationship for phenytoin in human plasma. The representative calibration curve for regression analysis and correlation coefficient (r^2) was greater than 0.99 in the concentration range of 101.253 to 10074.937 ng mL^{-1} for phenytoin.

Precision and accuracy: The precision of the assay was measured by the percent coefficient of variation over the concentrations of LLOQ, QC, LQC, MQC1, MQC2 and HQC samples during the course of validation. The accuracy of the assay was defined as the absolute value of the ratio of the calculated mean values of the LLOQ, low, middle and high quality control samples to their respective nominal values, expressed in percentage.

Absolute matrix effect: The matrix effect was assessed as recommended by Matuszewski [31] in 8 batches. The assessment of relative matrix effect was based on direct comparison of the MS/MS responses (peak areas) of the analytes spiked into extracts originating from different lots of plasma. The variability in these responses, expressed as CV %, was considered as the measure of relative matrix effect (Table-1).

Stability studies and dilution integrity: Stability experiments were performed to evaluate the analyte stability in stocks solutions and in plasma samples under different conditions, which may occur during sample analysis. Stock solution stability was performed by comparing area response of stability samples of analytes and the internal standard with the area response of sample prepared from fresh stock solutions. Bench-top stability, extracted sample stability (wet extract stability), freeze–thaw stability, dry extract stability and long-term stability were performed at LQC and HQC level using six replicates at each level. The dilution integrity experiment was intended to validate the dilution test to be carried out on higher analyte concentrations (above HQC), which may be encountered during real subject samples analysis.

Method development: Various mobile phase compositions of acetonitrile/methanol with acidic modifiers like formic

TABLE-1
MATRIX EFFECT OF PHENYTOIN IN HUMAN PLASMA

Plasma lot	LQC301.897			HQC 7549.310		
	Concentration found	Accuracy (%)	Internal standard - Normalized MF	Concentration found	Accuracy (%)	Internal standard - Normalized MF
1	304.415	100.83	1.00	7553.154	100.05	1.01
2	312.007	103.35	0.97	7565.46	100.21	1.01
3	314.494	104.17	0.95	7597.322	100.64	1.00
4	320.819	106.27	1.02	7792.341	103.22	1.01
5	317.578	105.19	1.01	7617.831	100.91	1.02
6	314.397	104.14	0.99	7513.575	99.53	1.00

acid, ammonium acetate and ammonium formate were tested in different volume ratios. Moreover various chromatographic columns like C8 and C18 of different make (Kromasil 100-5C_{18}, 100 × 4.6, 5 µm ; Alltima HP C_{18}, 50 × 4.6, 3 µm ; Zorbax SB C_{18}, 50 × 4.6, 5 µm; Zorbax XDB-phenyl 75 × 4.6, 3.5 µm; Ace 3C_{18}, 150 × 4.6, 3 µm; Hypurity advance 75 × 4.6, 5 µm; Discovery HS C_{18} 50 mm × 4.6 mm, 5 µm) were tested to achieve satisfactory retention time with a shorter run time, desirable separation from endogenous components, symmetric peak shape and adequate response for the analyte. The response obtained with acetonitrile and 0.1 % formic acid as the mobile phase was satisfactory. The eluted samples were chromatographed on zorbax Eclipse XDB-Phenyl 4.6 × 75 mm, 3.5 µm (Make: Agilent Technologies) column using a mobile phase consisting of HPLC grade acetonitrile: 0.1 % formic acid (80:20 v/v). The retention times of analyte and internal standard obtained with the above optimized chromatographic conditions were low enough (0.87 and 0.86 min) allowing a short run time of 2 min.

RESULTS AND DISCUSSION

Mass spectrometry: Phenytoin and internal standard were tuned in both positive and negative ionization modes initially, we opted ESI as the ionization source in the positive ionization mode for the method development as the response obtained in positive mode was much higher than in negative mode. The protonated form of analyte and internal standard, $[M+H]^+$ ion, was the precursor ion in the Q1 spectrum and was used as the precursor ion to obtain Q3 product ion spectra. The most sensitive mass transition was observed from *m/z* 253.20 (parent) and *m/z* 182.10 (product) for phenytoin and from *m/z* 263.20 (parent) and *m/z* 192.20 (product) for the internal standard (Fig. 2). The most intense and consistent product ion in Q3 MS spectra of the analyte and the internal standard was obtained by optimizing the collision energy and collision cell exit potential. The source parameters like nebulizer gas (GS1), auxiliary gas (GS2), collision gas, temperature and ion spray voltage were optimized to obtain adequate and reproducible response for the analyte. The dwell time for each transition was set at 200 ms.

Bioequivalence study: The study was an open, randomized, two-treatment, two-period, two-sequence, cross-over, single oral dose bioequivalence study with a two-week washout interval between the doses. After check-in, subjects received a standard meal (day 0), at dinner consisting of 1000-1200 k.cal after which they will be required to fast overnight (for at least 10 h) and a standard meals comprising of 2200-2400

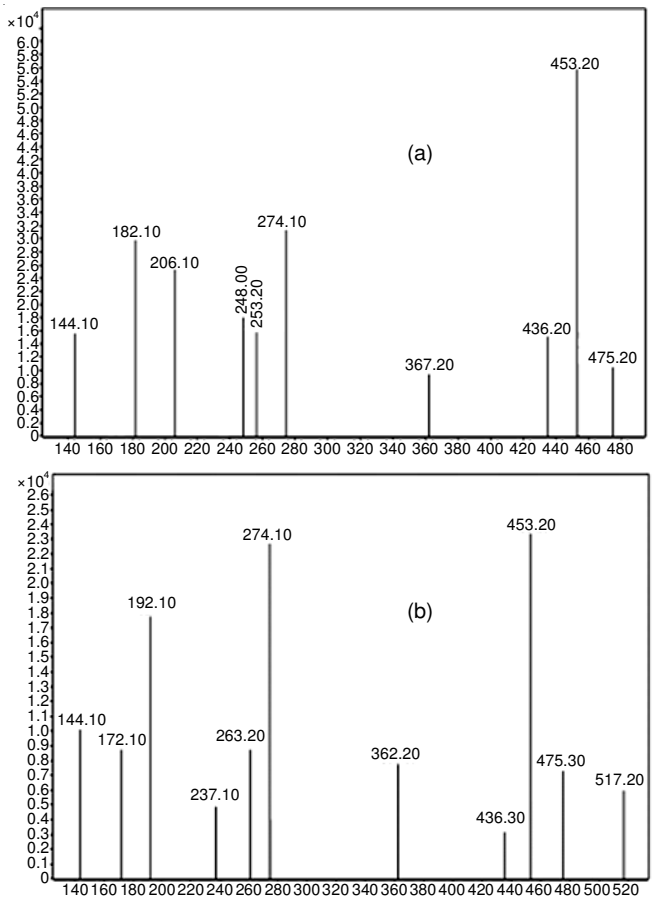

Fig. 2. Product ion mass spectra of (a) phenytoin and (b) phenytoin-d10

k.cal will be provided at 4, 8 and 13 h post-dose (*i.e.* lunch, snacks, dinner respectively) on day 1, a standard meal comprising of approximately 2400-2800 k.cal will be provided at 25, 29, 33 and 37 h post dose (*i.e.* breakfast, lunch, snacks and dinner respectively) on day 2, a standard meal comprising of approximately 2400-2800 k.cal will be provided at 49, 53, 57 and 61 h post dose (*i.e.* breakfast, lunch, snacks and dinner respectively) on day 3, a standard meal comprising of approximately 2400-2800 k.cal will be provided at 73, 77, 81 and 85 h post dose (*i.e.* breakfast, lunch, snacks and dinner respectively). Drinking water will be restricted for 1 h before and 1 h after investigational product administration except 240 ± 2 mL of water given for investigational product administration. Sixteen male healthy volunteers aged between 18 and 45 years and within 15 % of the ideal body weight were selected by clinical evaluation and laboratory tests [29,30]. The clinical protocol was approved by the local ethic committee and the

volunteers given written informed agreement to participate in the study. Twenty five blood samples (1 × 4 mL) will be collected in pre-labelled K2 EDTA vacutainers. Single venous blood sample will be withdrawn at pre-dose (0.00) and 0.50, 1.00, 2.00, 3.00, 4.00, 5.00, 6.00, 7.00, 8.00, 9.00, 10.00, 11.00, 12.00, 13.00, 14.00, 16.00, 20.00, 24.00, 36.00, 48.00, 72.00, 96.00, 120.00 and 144.00 h post-dose. The samples were immediately centrifuged (at 2500 rpm for 5 min at 4 °C), the plasma separated and kept frozen at -80 °C into labeled cryogenic tubes until assayed. A single oral dose of (three capsules) of phenytoin sodium 100 mg capsules (3 × 100 mg, a total of 300 mg) of test product (T) or phenytoin sodium flynn 100 mg hard capsules (3 × 100 mg, a total of 300 mg) of reference product (R) was administered as per the randomization schedule under fasting conditions. Each subject received the alternate 'treatment' in the subsequent period, in such a way that each subject will receive both the 'treatments' by the end of the study.

Selectivity: No interference was observed at retention time of phenytoin D10 when ULOQ concentration of phenytoin injected. Similarly, No significant interference was observed at retention time of phenytoin when working concentration of phenytoin D10 injected (Table-2).

The selectivity of the method was evaluated by analyzing a blank human plasma extract (Fig. 3A) and an extract spiked only with the IS (Fig. 3B). As shown in Fig. 3A, no significant direct interference in the blank plasma samples was observed from external substances in drug-free human plasma at the retention time of analyte and internal standard. Similarly, Fig. 3B shows the absence of direct interference from the internal standard to the MRM channel of analyte. Fig. 3C depicted a representative ion-chromatogram for the LLOQ QC sample (101.437 ng mL^{-1}). Typical MRM chromatograms of phenytoin and internal standard in human blank plasma (A), and human plasma spiked with internal standard (B), and a LLOQ QC sample along with internal standard (C) are shown in Fig. 3.

TABLE-2
INTER-DAY AND INTRA-DAY PRECISION AND ACCURACY FOR THE DETERMINATION OF PHENYTOIN IN HUMAN PLASMA

Quality control	Run	Concentration found (Mean ± SD ng mL^{-1})	Precision (%)	Accuracy (%)
Within batch Precision (five replicates at each concentration)				
LLOQ	1	100.9403 ± 4.40178	4.36	99.51
	2	97.8483 ± 7.12242	7.28	96.46
	3	100.0903 ± 5.44887	5.44	98.67
	4	102.4443 ± 5.83219	5.69	100.99
	5	98.3947 ± 3.05980	3.11	97.00
LQC	1	315.5153 ± 5.12792	1.63	104.51
	2	314.6637 ± 5.22432	1.66	104.23
	3	320.8195 ± 8.54987	2.67	106.27
	4	307.1255 ± 7.76445	2.53	101.73
	5	313.9517 ± 5.57732	1.78	103.99
MQC1	1	1563.0060 ± 27.72692	1.77	103.55
	2	1566.7513 ± 19.12779	1.22	103.79
	3	1561.1212 ± 24.48680	1.57	103.42
	4	1446.0695 ± 11.25903	0.78	95.80
	5	1486.2277 ± 32.24735	2.17	98.46
MQC2	1	4951.7305 ± 77.94930	1.57	98.41
	2	4959.5072 ± 113.23944	2.28	98.57
	3	4955.8138 ± 120.40596	2.43	98.49
	4	4880.3007 ± 102.22584	2.09	96.99
	5	4815.3207 ± 36.89935	0.77	95.70
HQC	1	7498.0583 ± 178.35924	2.38	99.32
	2	7566.7212 ± 158.63736	2.10	100.23
	3	7426.9042 ± 179.02481	2.41	98.38
	4	7525.2123 ± 144.14233	1.92	99.68
	5	7606.6138 ± 97.89235	1.29	100.76
Between batch precision (30 replicates at each concentration)				
LLOQ		99.9436 ± 5.25293	5.26	98.53
LQC		314.4151 ± 7.57551	2.41	104.15
MQC1		1524.6351 ± 55.04064	3.61	101.00
MQC2		4912.5346 ± 105.48331	2.15	97.63
HQC		7524.7020 ± 156.40757	2.08	99.67

Linearity: The representative calibration curve for regression analysis and correlation coefficient (r^2) was greater than 0.99 in the concentration range of 101.253 to 10074.937 ng mL^{-1} for phenytoin.

Precision and accuracy: Within-batch precision for LLOQ QC, LQC, MQC1, MQC2 and HQC ranged from 3.11-7.28, 1.63-2.67, 0.78-2.17, 0.77-2.43 and 1.29-2.41 %, respectively.

Between batch precision for LLOQ QC, LQC, MQC1, MQC2 and HQC was 5.26, 2.41, 3.61, 2.15 and 2.08 %, respectively.

Stability studies and dilution integrity: Using the mean of six replicates (n = 6), freeze thaw, wet extract, autosampler, bench top and short-term stability was assessed for stock solutions of phenytoin and internal standard. The per cent nominal ranged from 106.19 to 107.11 % and the precision ranged 1.05 to 1.46 % for four freeze thaw cycles. The results of wet extract stability studies demonstrate that the processed samples were stable for 29 h 39 min at 2-8 °C. The per cent nominal at 29 h 39 min ranged from 105.78 to 107.92 % and precision ranged from 1.16 to 2.00 %. The results of autosampler studies demonstrate that the processed samples were stable for 33 h 35 min. The percent nominal at 33 h 35 min ranged from 108.20 to 109.07 % and precision ranged from 1.36 to 1.99 %. The results of Bench top stability study shown that the percent nominal ranged from 100.79 to 103.86 % and the precision ranged from 1.48 to 2.48 % and The percent of short-term stability ranged from 97.55 to 98.12 % and the precision ranged from 0.70 to 1.83 % (Table-3).

Dilution integrity: Twelve sets of dilution integrity samples were prepared by spiking 1.60 time's of highest standard concentration (321.033 ng mL^{-1}). Six sets of dilution integrity samples were processed by diluting them twice and another six sets were processed by diluting them four times. These samples were analyzed along with a processed calibration curve standards (undiluted) of concentration range equivalent to that used for the calculation of precision and accuracy batch-3. The quality control sample concentrations were calculated using appropriate dilution factor. Phenytoin results demonstrate acceptable dilution integrity for two and four time's dilution. Phenytoin precision and accuracy, for a dilution factor of 2 was 0.70 and 101.88 %, respectively. Similarly, phenytoin precision and accuracy, for a dilution factor of 4 was 0.81 and 104.28 %, respectively.

Pharmacokinetics and statistical analysis: The pharmacokinetic parameters of phenytoin, namely the maximum plasma concentration C_{max} and time point of maximum plasma concentration T_{max} were obtained directly from the measured

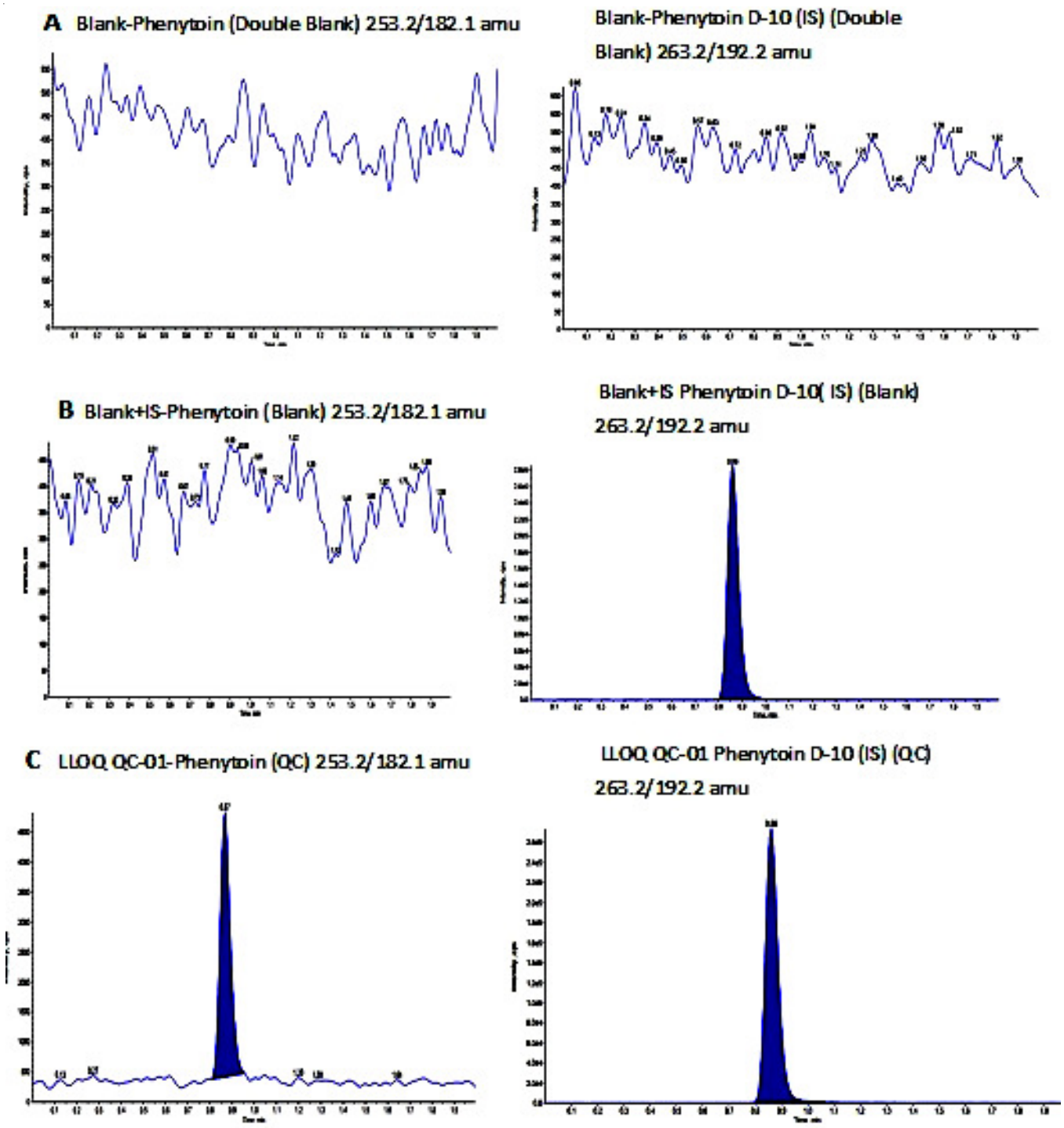

Fig. 3. Typical MRM chromatograms of phenytoin and internal standard in human blank plasma (A), human plasma spiked with internal standard (B), a LLOQ QC sample along with internal standard (C)

data. The area under the phenytoin plasma concentration time curve $AUC_{(0-t)}$ was computed using the linear trapezoidal rule, whereas, the area under the concentration plasma time curve from time 0 to the infinity $AUC_{(0-\infty)}$ was calculated as the sum of $AUC_{(0-t)}$ and C_t/k_e, where t was the time of the last measurable concentration (C_t) and k_e was the elimination rate constant. Fig. 4 depicts the mean plasma concentration *vs.* time profile of phenytoin after administration of a single oral dose of 100 mg (3 × 100; a total of 300 mg) of phenytoin under fasting conditions. The pharmacokinetic parameters of phenytoin were estimated with softwares using Phoenix WinNonlin 6.4 or higher version and Microsoft Excel version 7.0. After logarithmic transformation, $AUC_{(0-\infty)}$, $AUC_{(0-t)}$ and C_{max} values were subjected to analysis of variance (ANOVA), including the terms for subjects, treatment (sequence) and time period. The residuals of which were then tested for normality, as described by Chow & Liu [32]. For the evaluation of bio-equivalence, the point estimates and 90 % confidence interval (C.I.) for the relative difference between the test and reference formulations (test-reference) in each subject were constructed, using the residual mean

TABLE-3
STABILITY SUMMARY OF PHENYTOIN

Stability test	QC	Mean ± SD (ng mL^{1})	Accuracy/stability (%)	Precision (%)
Auto sampler stability (82 h, 30 min)	LQC	330.3717 ± 7.96866	109.43	2.41
	HQC	7737.4545 ± 147.88627	102.49	1.91
Wet extract stability (78 h, 25 min)	LQC	321.7963 ± 7.60493	106.59	2.36
	HQC	7752.6005 ± 152.51218	102.69	1.97
Bench top stability (6 h, 25 min)	LQC	333.2090 ± 9.52220	110.37	2.86
	HQC	7699.6332 ± 110.94605	101.99	1.44
Freeze–thaw stability (four cycles)	LQC	328.4350 ± 4.55586	108.79	1.39
	HQC	7671.8922 ± 81.56560	101.62	1.06
Reinjection stability (0 h)	LQC	315.5153 ± 5.12792	104.51	1.63
	HQC	7498.0583 ± 178.35924	99.32	2.38
Short-term stability (Freshly spiked QC's)	LQC	334.0085 ± 10.81075	110.62	3.24
	HQC	7704.4005 ± 87.19068	102.03	1.13
Short-term stability (at -20 °C for 6 days 12 h)	LQC	325.5962 ± 9.03039	107.85	2.77
	HQC	7757.6742 ± 130.37950	100.71	1.68

TABLE-4
MEAN PHARMACOKINETIC PARAMETERS FOR PHENYTOIN, AFTER A SINGLE 100 mg (3 × 100; A TOTAL OF 300 mg) ORAL DOSE ADMINISTRATION OF TEST AND REFERENCE FORMULATIONS TO SIXTEEN HEALTHY VOLUNTEERS

Parameter	Reference		Test	
	Mean	Standard deviation	Mean	Standard deviation
C_{max} (ng mL^{-1})	4543.5099	642.76876	4647.5213	580.61684
T_{max} (h)	6.667	3.658	18.143	29.4726
$T_{1/2}$ (h)	21.923	9.6836	19.576	6.4107
$AUC_{(0-t)}$ (ng h mL^{-1})	207221.7164	50321.50881	220141.2557	72272.70421
$AUC_{(0-\infty)}$ (ng h mL^{-1})	216178.6861	57786.4765	215180.267	58213.42536
Kel (1. h^{-1})	0.03681	0.014225	0.03929	0.013449

TABLE-5
GEOMETRIC MEAN OF THE INDIVIDUAL $AUC_{(0-t)}$, $AUC_{(0-\infty)}$ and C_{max} RATIOS (TEST/REFERENCE FORMULATION), THE RESPECTIVE 90 % CONFIDENCE INTERVALS (CI) AND POWER

Parameters	Geometric mean T/R ratio (%)	90 % Confidence interval		Power	Intra subject variability (relative standard deviation %)
		Lower	Upper		
log C_{max} (ng mL^{-1})	100.62	96.26	105.18	100	5.91
log AUC_{0-t} (ng mL^{-1})	103.72	97.46	110.38	99.96	8.31

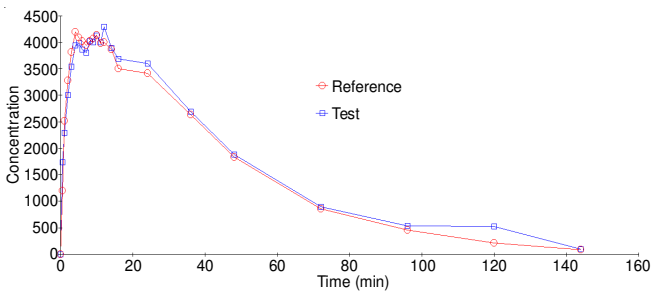

Fig. 4. Mean concentration time profile of phenytoin in human plasma after a single oral dose of 100 mg (3 × 100; a total of 300 mg) administration of test and reference formulations to 16 healthy volunteers

square error obtained from the multi-factorial ANOVA. The bioequivalence between the two formulations was evaluated based on the 90 % C.I. transformed back for the geometric mean ratios of $AUC_{(0-t)}$ and C_{max}, which are recommended within the 80-125 % (Table-5) interval according to the USFDA and EMEA guidelines.

Conclusion

The LC-MS/MS assay reported here is simple, rapid, specific and sensitive for quantification of phenytoin in human plasma and is fully validated according to commonly acceptable FDA and EMEA guidelines. The simple solid-phase extraction method gave consistent and reproducible recoveries for the analyte from human plasma. This is the first LC-MS/MS report for the determination of phenytoin based on the SPE technique for sample preparation. The stability of the analyte in plasma and in aqueous samples under different conditions has been extensively evaluated. A sample turnover rate of less than 2 min makes it an attractive procedure in high-throughput bioanalysis of phenytoin. This method was found to be reliable and reproducible to support pharmacokinetic studies in humans (Table-4). The 90 % confidence intervals were calculated for the C_{max}, $AUC_{(0-t)}$ and $AUC_{(0-\infty)}$, giving values between 96.26-110.38 % demonstrating the bioequivalence of the two formulations.

REFERENCES

1. D. Panomvana, N. Khummuenwai, S. Sra-lum and S. Towanabut, *Curr. Ther. Res.*, **68**, 325 (2007); https://doi.org/10.1016/j.curtheres.2007.10.007.
2. D. Blum, M. Reed and A. Metz, Prevalence of Major Affective Disorders and Manic/Hypomanic Symptoms in Persons with Epilepsy: A Community Survey, In: American Academy of Neurology, Denver, CO, USA (2002).

3. A. Ettinger, M. Reed and J. Cramer, *Neurology*, **63**, 1008 (2004); https://doi.org/10.1212/01.WNL.0000138430.11829.61.
4. M. Ha-Kaczyñska, *Pharmacol. Rep.*, **59**, ••• (2007).
5. C. Temiz, P. Temiz, A. Demirel, M. Sayin, A.S. Umur and F.D. Özer, *J. Clin. Neurosci.*, **16**, 307 (2009); https://doi.org/10.1016/j.jocn.2007.12.007.
6. C.M. Ohnmacht, S. Chen, Z. Tong and D.S. Hage, *J. Chromatogr. B Analyt. Technol. Biomed. Life Sci.*, **836**, 83 (2006); https://doi.org/10.1016/j.jchromb.2006.03.043.
7. N.J. Gogtay, S.S. Dalvi, R.B. Mhatre, B.G. Kirodian, A.H. Gupta, S.P. Jadhav and N.A. Kshirsagar, *Ther. Drug Monit.*, **25**, 215 (2003); https://doi.org/10.1097/00007691-200304000-00012.
8. H. Kodama, Y. Kodama, S. Shinozawa, R. Kanemaru, K. Todaka and Y. Mitsuyama, *J. Clin. Pharm. Ther.*, **23**, 361 (1998); https://doi.org/10.1046/j.1365-2710.1998.00173.x.
9. M.C. Meyer, A.B. Straughn, R.M. Mhatre, V.P. Shah, M.-L. Chen, R.L. Williams and L.J. Lesko, *Pharm. Res.*, **18**, 394 (2001); https://doi.org/10.1023/A:1011075502215.
10. M.R. Nuwer, T.R. Browne, W.E. Dodson, F.E. Dreifuss, J. Engel, I.E. Leppik, R.H. Mattson, J. Penry, D.M. Treiman and B.J. Wilder, *Neurology*, **40**, 1647 (1990); https://doi.org/10.1212/WNL.40.11.1647.
11. P.C. Lin, Y.H. Hsieh, F.F. Liao and S.H. Chen, *Electrophoresis*, **31**, 1572 (2010); https://doi.org/10.1002/elps.200900768.
12. E.J. Randinitis, R.A. Buchanan and A.W. Kinkel, *Epilepsia*, **31**, 458 (1990); https://doi.org/10.1111/j.1528-1157.1990.tb05503.x.
13. M. Mikati, N. Bassett and S. Schachter, *Epilepsia*, **33**, 359 (1992); https://doi.org/10.1111/j.1528-1157.1992.tb02328.x.
14. I. Soryal and A. Richens, *J. Neurol. Neurosur. Psych.*, **55**, 688 (1992); https://doi.org/10.1136/jnnp.55.8.688.
15. G.L. Lensmeyer, B.E. Gidal and D.A. Wiebe, *Ther. Drug Monit.*, **19**, 292 (1997); https://doi.org/10.1097/00007691-199706000-00009.
16. M.M. Bhatti, G.D. Hanson and L. Schultz, *J. Pharm. Biomed. Anal.*, **16**, 1233 (1998); https://doi.org/10.1016/S0731-7085(97)00265-3.
17. K.M. Patil and S.L. Bodhankar, *J. Pharm. Biomed. Anal.*, **39**, 181 (2005); https://doi.org/10.1016/j.jpba.2005.02.045.
18. R.H.C. Queiroz, C. Bertucci, W.R. Malfará, S.A.C. Dreossi, A.R. Chaves, D.A.R. Valério and M.E.C. Queiroz, *J. Pharm. Biomed. Anal.*, **48**, 428 (2008); https://doi.org/10.1016/j.jpba.2008.03.020.
19. T.A.C. Vermeij and P.M. Edelbroek, *J. Chromatogr. B Analyt. Technol. Biomed. Life Sci.*, **857**, 40 (2007); https://doi.org/10.1016/j.jchromb.2007.06.023.
20. M.J. Cwik, M. Liang, K. Deyo, C. Andrews and J. Fischer, *J. Chromatogr. B Biomed. Sci. Appl.*, **693**, 407 (1997); https://doi.org/10.1016/S0378-4347(97)00057-1.
21. Q. Fenghe, L. Li, L. Yi and L. Yongquan, *Se Pu*, **13**, 218 (1995).
22. S. Bardin, J.C. Ottinger, A.P. Breau and T.J. O'Shea, *J. Pharm. Biomed. Anal.*, **23**, 573 (2000); https://doi.org/10.1016/S0731-7085(00)00338-1.
23. S.M.N. Roy, S.M. Yetal, V.V. Vaidya and S.S. Joshi, *E-J. Chem.*, **5**, 169 (2008); https://doi.org/10.1155/2008/362512.
24. Y. Zhang, N. Mehrotra, N.R. Budha, M.L. Christensen and B. Meibohm, *Clin. Chim. Acta*, **398**, 105 (2008); https://doi.org/10.1016/j.cca.2008.08.023.
25. S.L. Dalmora, D.R. Nogueira, L.F. Londero, D.P. Santana and T.M. Goncalves, *Lat. Am. J. Pharm.*, **28**, 247 (2009).
26. M. Achim, D. Muntean, L. Vlase, I. Baldea, D. Mihu and S.E. Leucuta, *Stud. Univ. Babes-Bolyai Chem.*, **54**, 7 (2009).
27. D. Wang, X.Y. Chen and Y.F. Zhang, *Zhongguo Lin Chuang Yao Li Xue Za Zhi*, **22**, 213 (2006).
28. A. Zarghi, K. Gholami and M. Hessami, *Boll. Chim. Farm.*, **138**, 508 (1999).
29. FDA, Center for Drug Evaluation and Research, US Department of Health and Human Services, Guidance for Industry, Bioanalytical Method Validation (2001).
30. FDA, Center for Drug Evaluation and Research, US Department of Health and Human Services, Guidance for Industry, Bioavailability and Bioequivalence Studies for Orally Administered Drug Products–General Considerations (2002).
31. B.K. Matuszewski, *J. Chromatogr. B Analyt. Technol. Biomed. Life Sci.*, **830**, 293 (2006); https://doi.org/10.1016/j.jchromb.2005.11.009.
32. S.C. Chow and J.P. Liu, Design and Analysis of Bioavailability and Bioequivalence Studies, Marcel Dekker, New York, edn 2 (2000).