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Determination of Phenytoin in Human Plasma by a Validated Liquid Chromatography Method and its Application to a Bioequivalence Study

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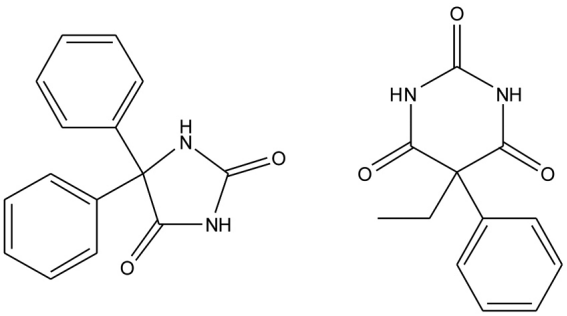
***SUMMARY*. A sensitive and specific method based on liquid chromatography was developed and validated for the determination of phenytoin in human plasma using phenobarbital as internal standard. The drugs were extracted from plasma by liquid-liquid extraction and separated isocratically on a Phenomenex Synergi MAX-RP C_{12} column (150 x 4.6 mm i.d.), with water: acetonitrile: methanol (58.8:15.2:26, v/v/v) as mobile phase. Detection was carried out using photodiode array detector set at 205 nm. The chromatographic separation was obtained within 12 min and was linear in the concentration range of 50-2500 ng/mL (r^2 = 0.9999). The method was successfully applied for the bioequivalence study of two tablet formulations (test and reference) of phenytoin 100 mg after single oral dose administration to 28 healthy human volunteers. The 90% confidence intervals were calculated for the C_{max}, $AUC_{(0-t)}$ and $AUC_{(0-\infty)}$, giving values between 99.97–118.40% demonstrating the bioequivalence of the two formulations.**

INTRODUCTION

Epilepsy is one of the most common serious central nervous system disorders, affecting 50 million people worldwide, and may cause persistent deformity and decrease quality of life [1]. Phenytoin (5,5-diphenyl-imidazoline-2,4-dione, Figure 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/day [2-4]. 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 [3,5]. Phenytoin has three pharmacologic characteristics associated with the risk of non-bioequivalence after the use as generic drug: poor water solubility, non linear dose-dependent pharmacokinetics, and a narrow therapeutic window [4,6].



(a) (b)

Figure 1. Chemical structures of phenytoin (**a**) and phenobarbital (**b**).

The reported analytical methods used to determine phenytoin concentrations in biological samples include liquid chromatography (LC) [3,4,7-16] and liquid chromatography-tandem mass spectrometry (LC-MS/MS) [17-19]. A reversed-phase LC (RP-LC) method with UV detection was applied for the bioavailability study of phenytoin capsules using liquid-liquid extraction with the long run time of 17 min [7]. Some others RP-LC methods were reported for the determination of phenytoin in pharmacokinetics and bioequiva-

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lence studies, but without detailed description of the method and the validation parameters [4,8-10]. 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 [11], protein precipitation extraction [12,13], stir bar-sorptive extraction [14], and solid-phase extraction using disposable cartridges with 1 mL sample volume capacity [15]. Moreover, the RP-LC method with UV detection was developed in the linear range from 0.4-400 µg/mL for the simultaneous determination of phenytoin and its prodrug fosphenytoin in human plasma and plasma ultrafiltrate [16]. Finally, LC-MS/MS methods for the quantitation of phenytoin in human plasma were developed and validated using negative ion atmospheric pressure chemical ionization [17] and positive electrospray ionization [18,19], with liquid-liquid and protein precipitation extraction, respectively.

The aim of the present work was to develop and validate a simple, precise and accurate LC method with UV detection, using liquid-liquid extraction, for the pharmacokinetic analysis of phenytoin in human plasma supporting the bioequivalence study of two pharmaceutical formulations.

MATERIALS AND METHODS

Chemicals and Reagents

The test and reference formulations containing 100 mg of phenytoin were manufactured by the Pharmaceutical Company Cristália, Batch 07064262 (Itapira, SP, Brazil) and Aventis Pharma, Batch 703176 (Suzano, SP, Brazil), respectively, within their shelf life period. Phenytoin reference substance (Batch J0E090) and phenobarbital (internal standard, I.S.) (Batch J) were purchased from United States Pharmacopeia (Rockville, MD, USA). HPLC-grade acetonitrile, methanol and tert-butyl methyl ether were obtained from Tedia (Fairfield, OH USA). All chemicals used were of pharmaceutical or special analytical grade. For all the analyses, ultrapure water was purified using an Elix 3 coupled to a Milli-Q Gradient A10 system (Millipore, Bedford, MA, USA). All solutions were filtered through a 0.45 µm membrane filter (Millipore, Bedford, MA, USA).

Apparatus and Chromatographic Conditions

A Shimadzu LC system (Shimadzu, Kyoto, Japan) was used equipped with a SCL-10AVP system controller, LC-10 AD_{VP} pump, DGU-14A degasser, CTO-10 AD_{VP} column oven, SIL-10AD-vp autosampler and a SPD-M10Avp photodiode array (PDA) detector. The detector was set at 205 nm and peak areas were integrated automatically by computer using a Shimadzu Class VP® V 6.14 software program. The experiments were carried out on a reversed phase Phenomenex (Torrance, USA) Synergi MAX-RP C12 analytical column (150 mm x 4.6 mm I.D., with a particle size of 4 µm and pore size of 80 Å) maintained at 35 °C. The LC system was operated isocratically using a mobile phase of water: acetonitrile: methanol (58.8:15.2:26, v/v/v). This was filtered through a 0.45 µm membrane filter (Millipore, Bedford, MA, USA) and run at a flow-rate of 1.2 mL/min. The injection volume was 50 µL for both standard and samples. The temperature of the autosampler was kept at 4 °C and the run-time was 12 min.

Standard Solutions and Calibration Curves

The stock solution of phenytoin was prepared by weighing 20 mg of reference material into a 10 mL volumetric flask and diluting to volume with methanol, obtaining a concentration of 2 mg/mL. Phenobarbital (I.S.) stock solution was made at a final concentration of 1 mg/mL using methanol. The prepared stock solutions were stored at 2-8 °C protected from light. An appropriate aliquot of phenytoin stock solution was diluted with methanol: water (10:90, v/v) to obtain the calibration standard solutions with the concentrations of 0.3, 0.6, 1.5, 3, 6, 9, 12 and 15 µg/mL. Then, 50 µL was taken of the standard solutions to prepare the analytical curves by spiking blank plasma at concentrations from 50 to 2500 ng/mL (50, 100, 250, 500, 1000, 1500, 2000 e 2500 ng/mL). A 6 µg/mL I.S. working solution was obtained by diluting the stock solution of phenobarbital with methanol: water (10:90, v/v). The quality control (QC) samples were prepared in blank plasma at concentrations of 150 (low), 1200 (medium) and 2200 ng/mL (high), and then divided in aliquots that were stored at -80 ºC until analysis. The spiked plasma samples (standards and quality controls) were extracted on each analytical batch along with the unknown samples.

Plasma Extraction

For the analysis of phenytoin, 300 µL of each plasma sample was transferred to a 15 mL glass tube, followed by addition of 50 µL of I.S. solution (6 µg/mL). All samples were mixed by vor-

tex agitation for 30 s. Then, a 4 mL aliquot of extraction solvent, tert-butyl methyl ether, was added using Dispensette Organic (Brand GmbH, Wertheim, Germany) and vortex-mixed for 90 s. The tubes were centrifuged for 15 min at 2700 rpm and the organic layer was filtered through a Millex GV 0.45 µm filter unit (Millipore, Bedford, MA, USA) into 15 mL conical tubes and evaporated under nitrogen stream while immersed in a 40 °C water bath. The residues were reconstituted with 300 µL of mobile phase. The samples were transferred to autosampler vials and 50 µL was injected into the LC system.

Validation of the Bioanalytical Method

The method was validated by the determination of the following parameters: specificity, linearity, range, recovery, accuracy, precision, lower limit of quantitation (LLOQ) and stability studies, following the bioanalytical method validation guidelines [20,21].

Specificity

Specificity was assessed using six blank human plasma samples, randomly selected, from different sources (including haemolysed and lipemic plasma), that were subjected to the extraction procedure and chromatographed to determine the extent to which endogenous plasma components could interfere in the analysis of phenytoin or the I.S. The results were compared to a solution containing 50 ng/mL of phenytoin.

Linearity and range

The analytical curves were constructed from a blank sample (plasma sample processed without IS), a zero sample (plasma processed with IS) and eight concentrations of phenytoin, including the LLOQ, ranging from 50 to 2500 ng/mL. The phenytoin/ I.S. peak area ratios were plotted against the respective standard concentrations to obtain the graph and the linearity evaluated by the least square regression analysis. The acceptance criteria for each calculated standard concentration was not more than 15% deviation from the nominal value, except for the LLOQ which was set at 20%.

Recovery

The recovery was evaluated by the mean of the response of three concentrations of phenytoin (150, 1200 and 2200 ng/mL), each one added of 1000 ng/mL of the IS, dividing the mean of the extracted sample by the mean of the unextracted sample (spiked with the extracted blank plasma residues) at the same concentration level.

Precision and accuracy

To evaluate the inter-day precision and accuracy, QC samples were analysed together with one independent analytical standard curve for 3 days, while intra-day precision and accuracy were evaluated through analysis of the QC samples in six replicates in the same day. Inter- and intra-day precision were expressed as relative standard deviation (RSD). The evaluation of precision and accuracy was based on the criteria [21] that the RSD of each concentration level should be within ± 15% of the nominal concentration.

Limit of quantitation

The lowest standard concentration on the analytical curve should be accepted as the limit of quantitation if the following conditions are met: the analyte response at the LLOQ should be at least five times the response compared to blank response and analyte peak (response) should be identifiable, discrete and reproducible with a precision of 20% and accuracy of 80-120%. The limit of detection (LOD) was defined by the concentration with a signal-to-noise ratio of 3.

Stability studies

The stability of phenytoin in human plasma was evaluated after each storage period and related to the initial concentration as zero cycle (samples that were freshly prepared and processed immediately). The samples were considered stable if the deviation (expressed as percentage bias) from the zero cycle was within ± 15%. The freeze-thaw stability of phenytoin was determined at low, medium and high QC samples (n = 3) over three freeze-thaw cycles within 3 days. In each cycle, the frozen plasma samples were thawed at room temperature for 2 h and refrozen for 24 h. After completion of each cycle the samples were analyzed and the results compared with that of zero cycle. The short-term stability was evaluated using three aliquots each of the low, medium and high unprocessed QC samples kept at room temperature (25 ± 5 °C) for 8 h, and then analysed. For the processed sample stability, three aliquots each one of the low, medium and high QC samples were processed and placed into the autosampler at 4 °C and analysed after 24 and 48 h. For the long-term stability analysis of phenytoin, three aliquots of each QC samples were frozen at –80 °C for 55 days and then analysed.

Bioequivalence Study

The study was an open, randomized, two period crossover design with a one-week washout interval between the doses. Twenty eight 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 [22,23]. The clinical protocol was approved by the local Ethic Committee and the volunteers given written informed agreement to participate in the study. During each period, a single oral dose of phenytoin (1 tablet-100 mg) was given with 300 mL of water after an overnight fast of at least 10 h. The dose chosen is clinically relevant as anti-convulsant drug in the treatment of epilepsy and it was expected to produce measurable plasma levels for a sufficient portion of the terminal elimination phase. Blood samples were collected at 0 h (pre-dose) and at: 0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 4.0, 5.0, 6.0, 7.0, 8.0, 9.0, 10.0, 12.0, 24.0, 48.0 and 72.0 h post dosing. The samples were immediately centrifuged (at 2500 rpm for 5 minutes at 4 °C), the plasma separated and kept frozen at -80 °C into labeled cryogenic tubes until assayed.

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 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 Ct/ke, where t was the time of the last measurable concentration (Ct) and ke was the elimination rate constant.

The pharmacokinetic parameters of phenytoin were estimated with softwares WinNonLin Professional Network Edition version 5.2.2.1 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 [24]. For the evaluation of bioequivalence, 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 square error obtained from the multifactorial ANOVA.

The bioequivalence between the two formulations was evaluated based on the 90% C.I. transformed back for the geometric mean ratios of $AUC_{(0-\infty)}$, $AUC_{(0-t)}$ and C_{max}, which are recommended within the 80-125% interval according to the guidelines [22,23].

RESULTS AND DISCUSSION

Method development

To obtain the best chromatographic conditions several columns with different bonded phases (C_{18} and C_{12}), and a number of parameters in composition of mobile phase were examined to provide sufficient selectivity, sensitivity and resolution in a satisfactory separation time. Phenytoin and I.S. showed similar retention on C_{18} and C_{12} column, and the C_{12} column was selected due to the separation of the interested peaks from plasma interferences. Mobile phases such as potassium phosphate buffer with different pH values and water were tested, and as no significant changes were obtained with the addition of buffer, water was chosen due to the simplicity and the lifetime of the column. The use of acetonitrile and methanol as organic modifiers improved the resolution, separation time and peak symmetry. For the selection of the best wavelength detection a PDA detector was used. The optimized conditions described for the LC method were used and validated for the analysis of phenytoin in human plasma, due to the capability and application to the bioequivalence study.

In order to produce a clean sample and avoid the introduction of non-volatile materials onto the column and LC system, liquid-liquid extraction was used for the sample preparation. Different organic solvents and mixtures were evaluated, including tert-butyl methyl ether, ethyl acetate, diethyl ether and dichloromethane, and tested different pH values, adjusted by the addition of formic acid, sodium hydroxide or buffer phosphate. The organic solvent tert-butyl methyl ether was used without pH adjustment, producing a chromatogram with no interfering peaks in drug-free plasma eluting at the retention times of interest, and also yielding the highest recovery for the analyte and I.S. compared to the others extraction solvents.

Phenobarbital was chosen as the I.S. as it showed a chromatographic behavior similar to that of the analyte and was completely resolved from it under the selected conditions. Representative chromatograms of a blank plasma, plasma spiked with phenytoin at 50 ng/mL (LLOQ) and plasma obtained from a human healthy volunteer 7 h after oral dose administration of phenytoin tablet 100 mg are presented in Figure 2. Typical standard retention times were 9.8 min

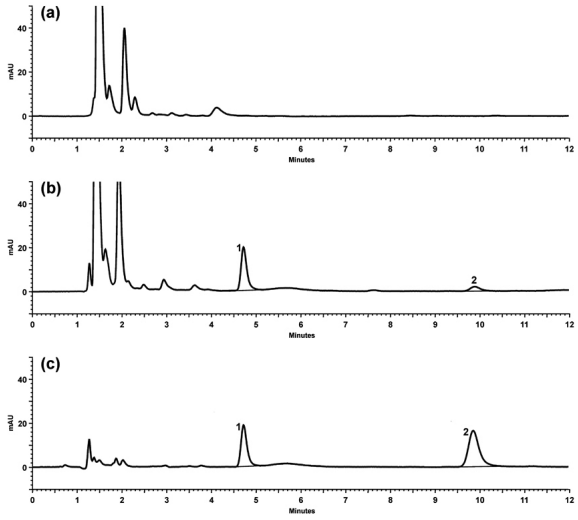

Figure 2. Representative chromatograms of: (**a**) Blank plasma sample; (**b**) Blank plasma spiked with phenytoin (LLOQ, 50 ng/mL) and 50 µL of phenobarbital (I.S., 6 µg/mL); (**c**) Plasma sample of a human healthy volunteer, collected 7 h after oral dose administration of phenytoin tablet 100 mg, and spiked with 50 µL of phenobarbital (I.S., 6 µg/mL). Peak 1 = phenobarbital, peak 2 = phenytoin.

for phenytoin and 4.7 min for I.S., and a back-pressure value of approximately 170 kgf was observed.

Method validation

Linearity was evaluated by six determinations of eight concentrations in the range of 50-2500 ng/mL. The values of the determination coefficient (r^2 = 0.9999, $y = 0.0014x - 0.0367$) indicated significant linearity of the analytical curves for the method. The LLOQ was evaluated in an experimental assay and was found to be 50 ng/mL with precision and accuracy lower than 20%. Comparison of the blank and spiked human plasma (50 ng/mL) chromatograms indicated that no significant interferences were detected from endogenous substances.

Phenytoin in human plasma was directly extracted with tert-butyl methyl ether by liquid–liquid extraction. The mean extraction recoveries for the three concentration levels of the QC samples were 96.98% for phenytoin and 96.01% for the internal standard, phenobarbital, showing the method suitability (Table 1).

Nominal Concentration (ng/mL)	% Recovery	
	Phenytoin [b] Mean (RSD [a] %)	Phenobarbital [b] Mean (RSD [a] %)
150	99.07 (6.87)	95.22 (1.91)
1200	95.95 (2.00)	96.06 (1.63)
2200	95.91 (0.75)	96.76 (1.11)
Mean	96.98 (1.81)	96.01 (0.80)

Table 1. Recovery of phenytoin and phenobarbital from human plasma after the extraction procedure. [a] RSD = Relative standard deviation; [b] Mean of six replicates.

The intra-day accuracy of the method was within 99.46 and 100.17% with a precision of 1.59-3.55%. The inter-day accuracy was within 98.71 and 100.07% with RSD of 1.51-4.48% (Table 2). The data show that the method possesses adequate repeatability and reproducibility.

Phenytoin was stable in neat plasma for up to 8 h at room temperature (short-term) and also after three freeze-thaw cycles, demonstrating that human plasma samples could be thawed and refrozen without compromising the integrity of the samples. Plasma samples were stable for at least 55 days at –80 °C (long-term). The results demonstrated that extracted samples could be analysed after keeping in the autosampler for at least 48 h with acceptable precision and accuracy. The results of stability of phenytoin in human plasma are shown in Table 3.

Bioequivalence and statistical analysis

The mean pharmacokinetic parameters after a single 100 mg oral dose administration of test and reference products to twenty eight healthy volunteers are presented in Tables 4 and 5.

No period effect was observed in the pharmacokinetic parameters studied (data not

Nominal Concentration (ng/mL)	RSD [a] (%)		Accuracy (%)	
	Intra-day [b]	Inter-day [c]	Intra-day [b]	Inter-day [c]
150	3.55	4.48	100.17	98.71
1200	1.59	1.51	99.46	98.98
2200	2.42	1.52	99.97	100.07

Table 2. Inter-day and intra-day precision and accuracy for the determination of phenytoin in human plasma. [a] RSD = Relative standard deviation; [b] Mean of six replicates; [c] Mean of three days.

Stability condition	150 ng/mL (average [a] ± RSD [b])	1200 ng/mL (average [a] ± RSD [b])	2200 ng/mL (average [a] ± RSD [b])
Fresh samples (zero cycle, %)	97.01 ± 4.22	96.47 ± 2.80	95.12 ± 2.25
Freeze-thaw stability (three cycles, -80 °C, %)	94.74 ± 2.26	98.27 ± 0.67	97.46 ± 0.89
Short-term stability (8 h, room temperature, %)	96.28 ± 3.51	96.58 ± 0.75	96.77 ± 2.01
Long-term stability (55 days, -80 °C, %)	91.53 ± 2.41	97.16 ± 0.85	98.34 ± 2.71
Post-preparative stability (48 h, 4 °C, %)	96.47 ± 1.67	98.05 ± 2.11	96.45 ± 2.27

Table 3. Stability of human plasma samples of phenytoin. [a]Mean of three replicates; [b]RSD = Relative standard deviation.

Parameter	Test		Reference	
	Mean	SD [a]	Mean	SD [a]
C_{max} (ng/mL)	1047.24	209.14	933.29	163.75
T_{max} (h)	11.07	6.49	12.89	8.13
$T_{1/2}$ (h)	16.36	3.13	17.67	4.21
$AUC_{(0-t)}$ (ng h/mL)	39408.74	8569.78	37701.90	8420.48
$AUC_{(0-\infty)}$ (ng h/mL)	42935.88	9545.26	42146.54	9226.47

Table 4. Mean pharmacokinetic parameters for phenytoin, after a single 100 mg oral dose administration of test and reference formulations to 28 healthy volunteers. [a] SD = Standard deviation.

Test/Reference	Parametric n = 28			
	Geometric	90% CI	Power	Intra-subject (RSD [a] %)
$AUC_{(0-t)}$ % ratio	104.67	100.87–108.62	1.00	8.13
$AUC_{(0-\infty)}$ % ratio	102.77	99.97-105.65	1.00	5.94
C_{max} % ratio	111.67	105.32-118.40	0.99	12.89

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. [a] RSD = Relative standard deviation.

shown). The curve of the mean phenytoin plasma concentration *versus* time obtained after a single oral dose of each phenytoin formulation is shown in Figure 3.

At any of the evaluation time, the mean values and the concentrations of phenytoin showed non significant differences between the individual subjects studied after the administration of each of the 2 formulations. The mean C_{max}, obtained at 11.07 and 12.89 h, were 1047.24 ng/mL and 933.30 ng/mL for test and reference formulations, respectively.

Further statistical analysis of pharmacokinetic variables that described the early and total exposure to phenytoin showed point estimates of the geometric means ratios of C_{max}, AUC_{0-t} and $AUC_{(0-\infty)}$ (phenytoin test vs. phenytoin refer-

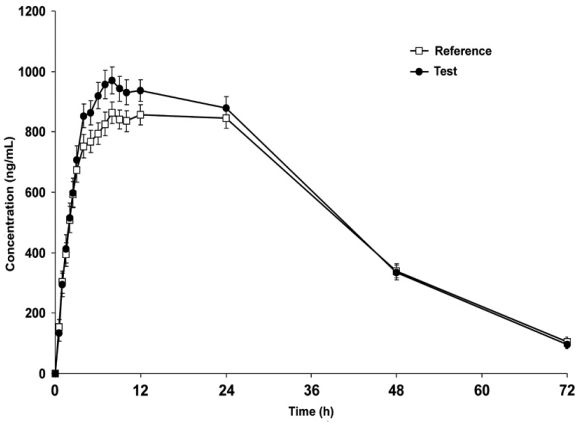

Figure 3. Mean plasma concentration – time profile of phenytoin after a single 100 mg oral dose administration to 28 healthy volunteers. Vertical bars indicate mean standard error.

ence) to be 111.67% (90% CIs: 105.32 – 118.40), 104.67% (90% CIs: 100.87 – 108.62) and 102.77% (90 % CIs: 99.97 – 105.65), respectively. For median T_{max} values, no significant differences ($P > 0.05$) were found between the two formulations evaluated.

CONCLUSION

A simple and accurate LC method, with a single step liquid-liquid extraction procedure, has been developed and validated for the determination of phenytoin in human plasma supporting a pharmacokinetic and bioequivalence study. The statistical analysis demonstrated that none of the parameters accepted for drug bioavailability ($AUC_{(0-t)}$, $AUC_{(0-\infty)}$, C_{max}) were significantly different between the treatments for the single dose data. Moreover, indicated that the two pharmaceutical products showed similar bioavailability profiles and therefore are considered bioequivalent with regard to the extent and rate of absorption and, interchangeable as well, for clinical and therapeutic purposes.

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