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BIOEQUIVALENCE STUDY OF TWO BRANDS OF PHENYTOIN SODIUM 100MG FORMULATIONS IN HEALTHY ADULT MALE RABBITS

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ABSTRACT

The objective of the study was to compare the bioavailability of a single oral 100 mg dose of two brands of phenytoin sodium formulations available in the Nepalese market. Formulation B was taken as test drug and compared with the innovator brand which was taken as reference standard. A randomized, two-way crossover study was done in six healthy adult male rabbits. All six rabbits received a single oral 100 mg dose of both the formulations with a two-week washout period between the formulations. Blood samples for plasma phenytoin levels were collected at 0.25, 1, 2, 4, 6, 8, 10, 12, 16, 24 hours. The pharmacokinetic parameters of the two brands of phenytoin sodium calculated were area under the concentration versus time curve from time zero to 24 hours (AUC 0–24), Area under the Curve from time zero to infinity (AUC0–∞), peak plasma concentration (C_{max}) and time of peak concentration (t_{max}). Formulation B failed to comply in terms of Area under the Curve (AUC), an important pharmacokinetic parameter to test bioequivalency, which was tested at significance level 0.05. This showed that the test formulation is not bioequivalent with the innovator. Taken together, our preliminary findings suggest that further studies in a large population is needed before switching phenytoin brands once a patient is carefully titrated to a given phenytoin brand.

INTRODUCTION: More than six decades after its introduction, problems of bioavailability and bioequivalence continue with phenytoin. Phenytoin has three pharmacologic characteristics associated with the risk of nonequivalence namely poor water solubility, nonlinear kinetics, and a narrow therapeutic window [1-2].

Absorption of phenytoin is highly dependent on the formulation of the dosage form. Particle size and pharmaceutical additives affect both the rate and extent of absorption.

Phenytoin is highly bound to plasma proteins and its metabolism is dose dependent. Its elimination follows first-order kinetics (fixed percentage of drug metabolized during a per unit time) at the low drug concentrations and zero-order kinetics (fixed amount of drug metabolized per unit time) at higher drug concentrations.

This change in kinetics reflects the saturation of metabolic pathways. Thus, very small increments in dosage may result in adverse effects [3].

Bioavailability is defined as the rate and extent (amount) of absorption of unchanged drug from its dosage form whereas bioequivalence is a relative term which denotes that the drug substance in two or more identical dosage forms, reaches the systemic circulation at the same relative rate and to the same relative extent i.e. their plasma concentration-time profiles will be identical without significant statistical differences [5-6].

Over the years, issues related to generic drug substitution has become a topic of broad discussion and hence bioavailability testing of a drug has been a topic of research interest. Bioavailability testing is an essential prerequisite for generic drug substitution and a method of predicting the clinical efficacy of a drug [2].

Since generic drug substitution is the process of dispensing a generic drug product in place of the prescribed drug product, it becomes mandatory that the substituted product must be a therapeutic equivalent to the prescribed product. Generic drug products are thus classified as therapeutic equivalents and expected to produce the same clinical effect and safety profile as the prescribed drug [4].

On the basis of above facts, the present study was undertaken to compare the bioavailability of a single oral 100 mg dose of two brands of phenytoin sodium formulations available in the Nepalese market.

MATERIALS AND METHODS:

Chemicals and Reagents: Phenytoin tablets (test formulation) with manufactured date june 2008 and expiry date may 2010 and phenytoin capsules (standard innovator formulation) with manufactured date april 2008 and expiry date march 2010 were obtained from local retail pharmacy. The reagents: water; acetonitrile; methanol used were of HPLC grade.

Phenytoin reference standard (potency: 99.95%) was obtained from Zest laboratories private limited, Kathmandu, Nepal. Hexobarbital internal standard was obtained from clinical pharmacology lab, Institute of Medicine, Kathmandu, Nepal.

Apparatus: All the glass apparatus were from Borosil; High Performance Liquid Chromatography was 5 PD-10 AVP from Shimadzu, Japan; Analytical balance was AW 220 from Shimadzu,Japan; Centrifuge apparatus was Labofuge 200 from Kendro laboratory products, Germany; Micropipette was Microlit, India; Disposable syringe 3ml was from Kana Laboratories, Korea and Disposable syringe 1ml was from Terumo, Japan.

Rabbits: Six adult healthy male rabbits of Angora species were used. The mean weight of the rabbits in kilograms was 2.523±0.282.The rabbits were divided into two groups and named as group 1 and group 2.

Method: Two ways cross over design was used for administration of either of the Phenytoin formulation. Group 1 received Standard formulation (Formulation A) whereas group 2 received Test formulation (Formulation B) during first study period. After a wash out period of two weeks group 1 received Formulation B and group 2 received Formulation A to complete the cross over design.

Capsule shells were removed from Formulation A and the powder were mixed with distilled water to give a concentration of 1 mg/ml. Similarly, Formulation B were crushed and mixed with distilled water to give a concentration of 1mg/ml. After an overnight fasting the rabbits were fed either of the formulations of Phenytoin 100 mg through disposable syringe 1 ml at a dose of 60 mg/kg. The rabbits were then given food and drink according to a standard schedule.

Approximately 2 ml blood samples were withdrawn at 15 mins, 1, 2, 4, 6, 8, 10, 12, 16, and 24 hours from marginal ear vein and centrifuged in tubes containing EDTA. The plasma was then separated by centrifugation (5000 rpm; 5 min) and stored at -10^{o}C until analysis were performed using HPLC. After a period of two weeks (i.e. washout period) the study was repeated in the same manner to complete the cross-over design.

Standard Curve preparation: 50 µL of blank plasma and 10 µL of hexobarbital (50 µg/ml) was taken and the volume of standard phenytoin solution was varied to give final concentration of 10 µg/ml to 60 µg/ml. 60 µL methanol was added each time to precipitate the plasma proteins.

After a brief vortex mixing the resulting solution was centrifuged for 5 min at 12000 rpm. The supernatant were then transferred to another set of clean tubes and a 10-20 µL aliquot were injected into the chromatographic system.

The linearity range was then established from 10 µg/ml to 60 µg/ml by using linear regression analysis which gave the equation of a straight line in the form of:

$y = 0.00351 + 0 .04161 x$ $(y = a+ bx)$----------(i)

Where y is the ratio of standard drug area to that of hexobarbital area and x is the standard concentration. a is the y intercept and b is the slope of the straight line . The correlation coefficient (r) was found to be 0. 953.

Sample analysis: To 50 µL of test plasma sample, 10 µL of hexobarbital (as internal standard) (50 µg/ml in acetonitrile) and 30 µL of phenytoin standard (50 µg/ml) and 60 µL of methanol were added. After a brief vortex mixing, it was centrifuged for 5 min at 12,000 rpm. The supernatant were then transferred to another set of clean tubes and a 10-20 µL aliquot were injected into the chromatographic system. The chromatographic conditions consisted of mobile phase of acetonitrile and water in the ratio of 25:75 respectively. Flow rate of 1.5 mL/min was maintained through the column (C_{18}). The peaks were monitored at a wavelength of 210 nm at 35^{o}C.

Pharmacokinetics study: The ratio of drug area to hexobarbital area was then fitted in above equation (i) to get the values of drug concentration at various time intervals. From the concentrations thus obtained Area under the plasma concentration time curve from time zero to twenty four hours ($AUC_{0–24}$) was calculated by trapezoidal rule where 24 hours was the last time point. Area under the plasma concentration − time curve from time zero to time infinity ($AUC_{0–\infty}$) was calculated as:

$(AUC_{0–\infty}) = AUC_{0–24} + C_{24}/K_e$ ----------(ii)

Where C_{24} was the last drug concentration measured and K_e was the elimination rate constant calculated from the slope of the line plotted as log concentration versus time.

Absorption rate constant K_a was calculated by Wagner Nelson method. A graph was plotted between log percentage Amount remaining to be absorbed (ARA) versus time and the slope of the straight line obtained times – 2.303 gave the K_a.

Time of peak concentration (t_{max}) was then calculated by using the following formula:

$$T_{max} = \frac{2.303 \log (Ka / Ke)}{Ka – Ke}$$

Where Ka and Ke are absorption rate constant and elimination rate constant respectively.

Then the value of FXo/ Vd was calculated using following formula:

$Ct = FXo/ Vd * Ka/ Ka – Ke (e^{-Ket} – e^{-Kat})$ ----------(iii)

Where Ct is concentration at certain time t.

From the values of FXo/ Vd Peak Plasma Concentration (C_{max}) was then determined by using following formula:

$Cmax = FXo / Vd\ e^{- Ke\ Tmax}$ ----------(iv)

Validity and Reliability: The method was developed after going through original research articles published in international peer reviewed, indexed journals. A pilot study involving phenytoin formulation (Eptoin tablets) on two rabbits were done before the final experiment. The study was approved by the Ethical Review Board for Research of the Institute of Medicine, Tribhuvan University.

Statistical Analysis of Data: Both univariate and bivariate analysis was used for analysis of data. In univariate analysis mean, standard deviation, confidence interval etc were used. Bivariate analysis was done by using student's t- test. The value set for statistical significance was $p< 0.05$ using two-sided test. All the analysis was carried out by using Microsoft excel 2003 and STATS version 1.1 1998.

RESULTS: Area under the Curve from zero to 24 hour. Area under the Curve from zero to 24 hour of the two formulations were calculated individually and are summarized in **table 1**.

TABLE 1: AREA UNDER THE CURVE FROM ZERO TO 24 HOUR OF THE TWO FORMULATIONS

Rabbit	Formulation A* (mcg/mL hr)	Formulation B* (mcg/mL hr)
R1	258.93	466.32
R2	546.22	488.78
R3	250.79	205.62
R4	481.67	332.26
R5	380.50	312.07
R6	287.80	389.37
Mean ± SD	367.65 ± 124.02	365.74 ± 105.32
SE	50.62	42.98

*Different from each other at significance level 0.05, p=0.0224

Area under the curve from zero to 24 hour of two different formulations is shown in table 1. The area under the curve of two formulations was significantly different from each other at significance level 0.05 (p = 0.0224). Maximum AUC was shown by R2 for both the formulations.

Area Under the Curve from zero to infinity: Area Under the Curve from zero to infinity of the two formulations was calculated individually and is summarized in **table 2**.

TABLE 2: AREA UNDER THE CURVE FROM ZERO TO INFINITY OF THE TWO FORMULATIONS

Rabbit	Formulation A*(mcg/mL hr)	Formulation B* (mcg/mL hr)
R1	271.81	552.22
R2	607	664.31
R3	257.29	222.20
R4	546.77	349.44
R5	504.85	338.13
R6	306.13	404.49
Mean ± SD	415.64 ± 154.61	421.80 ± 160.12
SE	63.12	65.37

*Different from each other at significance level 0.05, p=0.0527

Area under the curve from zero to infinity of two different formulations is shown in table 2. The area under the curve of two formulations was significantly different from each other at significance level 0.10 (p = 0.0527). Maximum AUC was shown by R2 for both the formulations.

Time of Peak Concentration (t_{max}): Time of Peak Concentration (t_{max}) of the two formulations was calculated individually and is summarized in **table 3**.

TABLE 3: TIME OF PEAK CONCENTRATION (T_{MAX}) OF THE TWO FORMULATIONS

Rabbit	Formulation A* (hr)	Formulation B* (hr)
R1	10.26	10.42
R2	8.74	7.37
R3	4.05	7.70
R4	7.82	4.47
R5	6.11	11.8
R6	8.15	6.46
Mean ± SD	7.52 ± 2.17	8.04 ± 2.67
SE	0.88	1.09

*No difference from each other at significance level 0.05, p=0.2811

Time of Peak Concentration (t_{max}) of two different formulations is shown in table 3. The time of Peak Concentration (t_{max}) of two formulations was not significantly different from each other at significance level 0.05. (p = 0.2811). Maximum time for peak concentration was shown by R1 for formulation A whereas the time for maximum peak concentration was found in R5 for formulation B.

Peak Plasma Concentration (C_{max}): Peak Plasma Concentration of the two formulations were calculated individually and are summarized in **table 4.**

TABLE 4: PEAK PLASMA CONCENTRATION (CMAX) OF THE TWO FORMULATIONS

Rabbit	Formulation A# (mcg/mL)	Formulation B# (mcg/mL)
R1	26.05	20.19
R2	24.20	30.60
R3	32.13	26.0
R4	29.28	31.58
R5	22.56	35.54
R6	28.34	26.71
Mean ± SD	27.09 ± 3.51	28.44 ± 5.33
SE	1.43	2.17

No difference from each other at significance level 0.05, p=0.3844

Peak Plasma Concentration (Cmax) of two different formulations is shown in table 4. The Peak Plasma Concentration (Cmax) of two formulations was not significantly different from each other at significance level 0.05. (p= 0.3844). Maximum plasma concentration was shown by R3 for formulation A whereas the maximum concentration was found in R5 for formulation B.

DISCUSSION: Assessment of bioequivalence of local product to innovator product is required to exclude any clinically important differences in the rate or extent at which the active entity of the drugs becomes available at the site of action. Two drugs are considered to be bioequivalent if they are pharmaceutically equivalent and their bioavailability is so similar that they are unlikely to produce clinically relevant differences in regard to safety and efficacy [7].

In the present two-way randomized crossover study carried out in six healthy adult male rabbits, comparison of the bioavailability of one brand of phenytoin sodium 100 mg formulation with innovator formulation of same strength was done. Both drugs were purchased from the retail pharmacy by the investigator, and the formulation was administered orally in a single 60 mg/kg dose in the fasting state. During Pharmacokinetic assessment, the Area Under the Curve was significantly different at significance level 0.05 while there was no significant difference between two formulations in case of C_{max} and T_{max} at significance level 0.05.

AUC zero to 24 hrs was found to range from 250.79 to 546.22 µg/ml hr and AUC zero to infinity was in range 257.29 to 607 µg/ml hr in formulation A whereas AUC zero to 24 hrs was found to range from 205.62 to 488.78 µg/ml hr and AUC zero to infinity was in range 222.20 to 664.31 µg/ml hr in formulation B. This result showed that there was a wide variation in AUC from zero to 24 hrs and AUC from zero to infinity between rabbits. This variation observed might be due to physiological variations between rabbits used in the study.

In a study done by Gogtay et al. it was also seen that there is wide variations between AUC of the same phenytoin formulation in healthy volunteers [8].

The bioavailability of drugs is determined by gastrointestinal physiological factors and the physicochemical property of drugs. The presence of food changes the physiological functions of gastrointestinal mucosa (*e.g.*, gastric *p*H, gastric emptying, hepatic blood flow) which may lead to increase or decrease in the bioavailability of drugs. These factors can change pharmacokinetic parameters used in assessing bioavailability [9-10].

The present study in rabbits showed higher peak plasma concentration 27.09±3.51 µg/ml for formulation A and 28.44±5.33 µg/ml for formulation B than those seen by other researchers. In a similar study done by Soma et al. to determine pharmacokinetics of phenytoin in horses, peak plasma concentration observed was 11.8±0.68 µg/ml [11]. This might be due to the fact that phenytoin is highly permeable and there might be diluents like binders added in small amounts in the formulation. Due to no binding of drug to the diluent, a larger amount of drug might have released from the formulation. Minain et al. carried similar study in rats and Cmax observed was 7.82 µg/ml [12].

Similarly, time of peak concentration found in the present study in rabbits was higher compared to similar studies done in the past. In a study done by Gogtay *et al*., time of peak concentration observed was quite lower [8]. Time of peak concentration is related to the rate of absorption and fasting state of rabbits might have resulted in higher time of peak concentration. The formulation was administered as a solution; it could also have resulted in faster onset time with corresponding higher time of peak concentration.

CONCLUSION: The bioequivalence study of two formulations of phenytoin sodium 100 mg available in Nepalese market showed that the test formulation is not bioequivalent with the innovator. The test formulation was not significantly different in peak plasma concentration and time of peak concentration but it was significantly different in terms of Area Under the Curve which is the most important parameter in evaluating the bioavailability of a drug from its dosage form as it represents the extent of absorption. As phenytoin is one of the commonly used drugs in the treatment of primary and secondary generalized tonic-clonic epilepsy in Nepal having narrow therapeutic index, switching phenytoin brands could have significant implications and is not advisable once a patient is carefully titrated on one formulation.

Limitations of Study: The main drawback of this study is that it is done only in a small number of animals. Though similar environmental conditions and feed is provided to rabbits during the study period, there might be considerable differences when the drug is used in humans.

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