BIOPHARMACEUTICS & DRUG DISPOSITION, VOL. 12, 687–695 (1991)

BIOAVAILABILITY STUDY OF A FREEZE-DRIED SODIUM PHENYTOIN-MILK FORMULATION

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KEY WORDS Bioavailability Phenytoin Milk Freeze drying Solubility Binding

INTRODUCTION

The problematic bioavailability of phenytoin's (5,5-diphenylhydantoin) oral formulations serves as a stimulus for examining new formulations and/or administration conditions that may provide more predictable absorption. Attempts to achieve more consistent peroral phenytoin bioavailability from conventional solid dosage forms include changes of binder and crystal size, use of salt form, and inclusion of the drug in cyclodextrins.[1–3] In addition, various factors which may affect the environment and/or the physiology of the upper gastrointestinal tract can profoundly affect the absorption of phenytoin.[1,4,5] Among other approaches, the use of drug–milk freeze-dried formulations has been proposed to overcome problems associated with dissolution-limited bioavailability.[6,7] The effect has been attributed to the formation of an amorphous precipitate during the drying process which facilitates the re-dissolution of the drug during the regeneration of the milk solution.[6,7] In this work, we report comparative bioavailability studies utilizing a freeze-dried sodium phenytoin–milk formulation and a capsule formulation administered with either water or milk. In addition, the interaction of the drug with milk components was evaluated *in vitro* through binding and solubility studies.

EXPERIMENTAL

In vitro studies

Binding studies. The binding of sodium phenytoin to milk components was studied as a function of temperature and fat content by equilibrium dialysis using 5 ml cells (Dianorm system, Bioblock Scientific). Three temperatures (15, 25, and 37°) and two types of milk (Long Life Milk, Landgenossenschaft,

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0142–2782/91/090687–09$05.00

Received 4 February 1991
Revised 14 June 1991

Ennstal, Stainach, Steiermark, Austria) in terms of their fat content (i.e. 0·75 and 3·5 per cent) were utilized. The donor compartment contained the drug in phosphate buffer (0·2 M, pH 6·6) while milk was the only content of the receiver compartment. Due to solubility limitations, initial drug concentrations in the buffer compartment varied with temperature and were 5, 10, and 18 $\mu g\ ml^{-1}$ (for the 15° experiments), 7, 10, and 20 $\mu g\ ml^{-1}$ (at 25°) and 10, 20, and 30 $\mu g\ ml^{-1}$ (at 37°). At equilibrium, drug concentrations in both compartments were measured. At each temperature and for each milk type there were nine phenytoin binding determinations, i.e. triplicate determinations at each of three phenytoin concentrations.

Solubility studies. The solubility of sodium phenytoin in phosphate buffer (0·2 M pH 6·6) and milk was studied as a function of temperature and the fat content of milk by using a shaking water bath (Julabo SW1, Schwarzwald, Germany). Both temperature and fat content ranges were similar to those utilized in the binding studies. Samples were filtered through double Whatman® (no. 42) papers into an appropriate diluent depending on the nature of the sample (milk or buffer). Solubility was determined in triplicate at each temperature and for each medium.

Assay and data analysis. Concentrations of sodium phenytoin in both milk and aqueous solutions were measured with HPLC using carbamazepine as internal standard. Aqueous samples were diluted with a methanolic solution of carbamazepine (1:1), vortexed, and injected into the chromatograph (20 µl in a Rheodyne injector model 7125). The milk assay was accomplished by a modified method for measuring phenytoin serum concentrations.[8] The sample (0·5 ml) was diluted with 5 ml dichloroethane containing the internal standard. While vortexing, 0·5 ml hydrocloric acid (1N) was added dropwise to ensure denaturation of casein micelles and protein aggregation. The samples were then centrifuged and 2 ml of the organic layer was transferred into another test tube and dried under nitrogen at 50°. The residue was dissolved in methanol (2–3 ml depending on the type of the experiment) and 20 µl were injected into the chromatograph. The recovery of the drug from 10 milk samples spiked with various concentrations of sodium phenytoin was found to be (mean ± SD) 100·6 ± 1·4 per cent. The detection limit of the method was 250 $ng\ ml^{-1}$. The intra-day variability was tested using six samples of 4 $\mu g\ ml^{-1}$ and six samples of 25 $\mu g\ ml^{-1}$ and found to be 2·7 and 2·2 per cent, respectively; the inter-day variability (six samples of 30 $\mu g\ ml^{-1}$) was 4·7 per cent. The HPLC system consisted of a Spectra Physics® (San Jose, CA, USA) model SP 8800/8810 LC pump, a model Spectra 100 UV detector set at 250 nm coupled with an SP 4400 Integrator and a Spherisorb S10 ODS2 column (15 cm × 4·6 mm) thermostated (Eldex, San Carlos, CA, USA, model CH-150) at 50 ± 0·1°. The mobile phase consisted of 10 per cent acetonitrile, 34 per cent methanol, and 56 per cent water containing 0·04 per cent n-butylamine and 0·04 per cent

glacial acetic acid. The flow rate was 1·6 ml $min^{-1}$with resulting retention times 6·1 and 7·4 min for phenytoin and carbamazepine, respectively.

Differences were considered to be statistically significant at the 0·05 level and assessed by one-way analysis of variance (ANOVA). ANOVA was followed by Tukey's procedure to identify which means are different from one another.

The effect of fat content on the per cent bound at a specific temperature was evaluated by the unpaired *t*-test.

In vivo studies

Preparation of test formulations. The method described previously[6,7] was employed to prepare the freeze-dried formulations. A volume of 10 ml of sodium phenytoin (Minerva, Athens, Greece; lot no. 4697) solution in 0·1 N NaOH was added in a dropwise manner with stirring to 200 ml of low-fat milk (fat concentration 0·75 per cent). The pH values of the final solutions were not altered significantly (pH range 6·8–7·2). The resulting solutions were further lyophilized (Secfroid, Lausanne, Switzerland), and the dry material was collected and kept in airtight amber glass bottles.

Preparation of control formulations. A quantity of 200 mg of pure drug substance was placed in a size 0 gelatin capsule to make the control formulation.

Bioavailability study. Four healthy male volunteers with age and weight ranges 28–41 years and 64–75 kg, respectively, participated in the study. Each subject underwent physical examination and after receiving extensive information about the study gave written consent. All subjects refrained from alcohol and any drug for at least 48 h prior to drug administration as well as during the investigation. Individual subjects were assigned formulations according to the three-period randomized crossover design. Washout periods were 3 weeks.

On three separate occasions, each volunteer received the regenerated (with 200 ml of water) sodium phenytoin–milk solution, a capsule of 200 mg sodium phenytoin with 200 ml of low fat milk and a capsule of 200 mg sodium phenytoin with 200 ml of water. To counteract the bitter taste of the drug, 20·0 mg of sodium saccharin (Serva Feinbiochem, GMBH and Co.) were dissolved in all fluids. All subjects continued to fast for 4 h after the administration of the formulations. Blood samples were collected (Butterfly, 21INT, Abbott Laboratories) at 0·5, 1·0, 1·5, 2·0, 2·5, 3·0, 4·0, 6·0, 8·0, 12·0, and 24·0 h after the administration of the formulations. Serum samples were stored (for not more than 48 h) at −30° until analysed.

Assay and data treatment. Serum concentrations of phenytoin were determined by a fluorescence polarization immunoassay (FPIA) method, using the

Abbott TDx system.[9] The lowest measurable concentration that can be distinguished from zero at the 95 per cent confidence level was 0·5 μg ml^{-1}.

Pharmacokinetic analysis was performed by fitting an open one-compartment model with linear kinetics to each subject's data using the PCNONLIN® program.[10] Initial estimates for the absorption rate constant (k_a) and the elimination rate constant (k_e) were calculated by the method of residuals.

Statistically significant ($p = 0{\cdot}05$) differences between the pharmacokinetic parameters were evaluated with one way ANOVA coupled with Tukey's procedure to determine the differences among the treatments.

RESULTS

In vitro studies

The binding of the drug with milk components was found to be not dependent on the concentration of the drug at all temperatures studied. The mean percentage of phenytoin binding to milk components is given in Table 1. For both types of milk, binding decreases as temperature increases. However, only at the highest temperature (37°) are the values significantly less than those observed at the two lower temperatures. In addition, elevated fat content contributed consistently to a small ($\approx$ 10–12 per cent) but significant increase of the percent bound.

Table 1. Average per cent bound phenytoin to milk components at various temperatures using two types of milk[1]

Temperature (°)	Fat content 0·75%	3·5%
15	63·1 (2·6)	69·2 (3·1)
25	58·8 (3·8)	66·1 (2·6)
37	52·3 (4·4)	58·9 (2·4)

[1] Mean (SD) from nine experiments.

Data on the effect of temperature on the solubility of sodium phenytoin in an aqueous medium having the same pH as milk (0·2 M phosphate buffer pH 6·6) and in milk are shown graphically in Figure 1. Solubilities in both milk types were always significantly higher than those observed in phosphate buffer (pH 6·6); fat content did not affect these differences. The biggest differences (14-fold) between buffer and milk were observed at the lower temperatures whereas at 37° there was only an 8·5-fold increase in solubility. Further, the solubility of the drug increased with temperature; however, the increase in the aqueous medium was significant only between 25 and 37° (approx. 70 per

cent), while in milk, a significant difference was observed only from 15 to 25° (approx. 40 per cent).

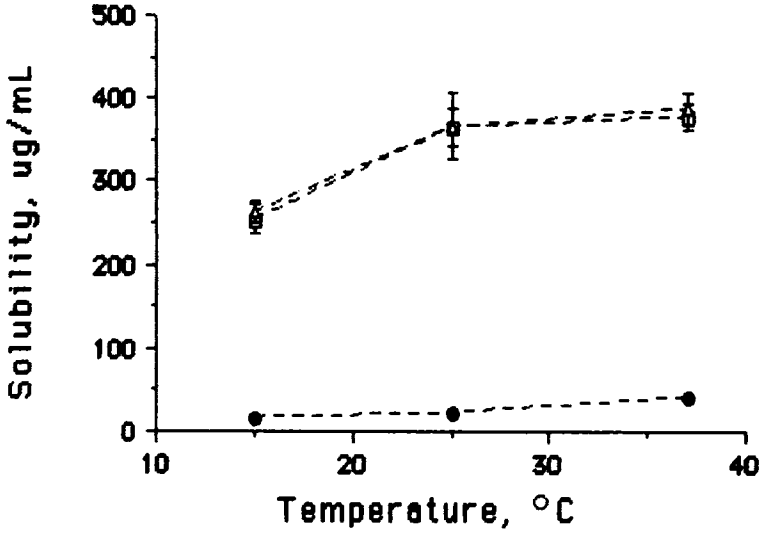

Figure 1. Solubility (mean ± SD) of sodium phenytoin as a function of temperature. Key: ●: phosphate buffer (pH 6·6, 0·2 M); □: low-fat milk (fat content 0·75 per cent); △: high fat milk (fat content 3·5 per cent)

In vivo studies

Figure 2 shows the mean serum phenytoin concentration versus time relationship for four subjects following the administration of the three oral formulations. Although Michaelis-Menten kinetics are undoubtedly applicable when repeated phenytoin doses are administered, it is debatable whether this is the case for a single dose administration[11,12] and especially when serum concentrations are very low (<4 μg ml^{-1}).[13] By fitting an open one-compartment model with Michaelis-Menten elimination kinetics to the data, consistently poor correlation coefficients were obtained and in some cases convergence was impossible. Therefore, the analysis was performed assuming linear kinetics. Correlation coefficients were always significant and ranged from 0·883 to 0·983. Mean (SD) values for all pharmacokinetic parameters estimated from each subject's data are presented in Table 2. One-way ANOVA indicated significant differences between test and control formulations only for the absorption rate constants and the time to peak serum concentration. Tukey's procedure showed significant differences only between the test formulation and the milk control formulation. The extent of drug absorbed (relative bioavailability) from the freeze-dried formulations is not significantly different from that of the control formulations.

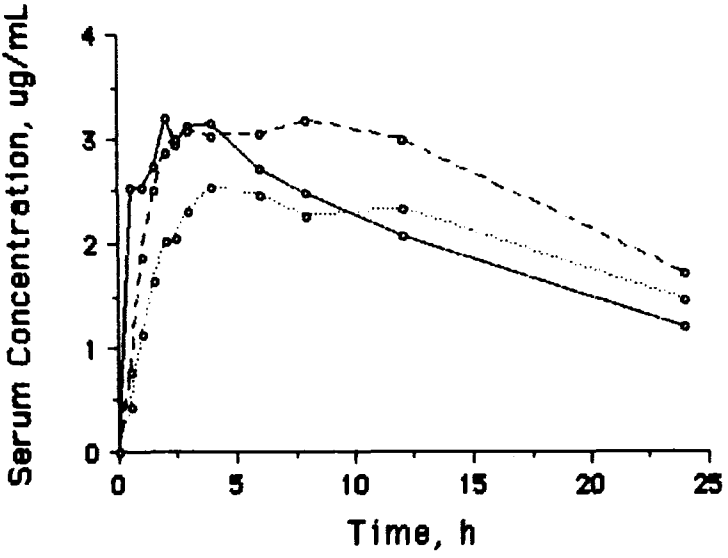

Figure 2. Mean serum phenytoin vs time concentrations after administration of 200 mg of sodium phenytoin in four subjects. CV of each experimental point ranged from 5 to 41 per cent. Key: ——: 200 ml of test formulation; -----: capsule with 200 ml of water; ·····: capsule with 200 ml of milk

Table 2. Mean (SD) pharmacokinetic parameters for phenytoin estimated with PCNONLIN® following the administration of test and control formulations to four subjects

Parameter	Test	Control with water	Control with milk
FD/V ($\mu g\ ml^{-1}$)	3·6 (0·5)	3·9 (1·0)	3·4 (0·7)
K_a (h^{-1})	2·76 (1·91)	0·71 (0·28)	0·44 (0·12)
K_{el} (h^{-1})	0·05 (0·02)	0·03 (0·02)	0·04 (0·02)
t_{max} (h)	2·2 (1·6)	4·9 (1·1)	6·1 (1·5)
C_{max} ($\mu g\ ml^{-1}$)	3·2 (0·4)	3·3 (0·7)	2·6 (0·5)
AUC ($\mu g\ h\ ml^{-1}$)	78·4 (20·9)	151·2 (72·2)	81·9 (21·3)

DISCUSSION

Both the observed non-specific binding of phenytoin to milk components and the increased binding at lower temperatures agree with previous data using several drugs.[14] Moreover, the effect of fat content in milk is consistent with the lipophilicity of phenytoin (the 1-octanol-water *logP* value is 2·47)[15] and other drugs with similar *logP* values which exhibit similar binding behaviour to milk components.[14] As expected, the solubility of sodium phenytoin in pH 6·6 buffer was somewhat lower than the values reported earlier using pH 7 buffer[16] and higher than reported for pH 1.[17] The substantially greater solubi-

lity in milk, compared to the aqueous buffer, is also in agreement with a previously established relationship. The latter[14] indicates that the less soluble a drug is in pH 6·6 buffer the higher will be the increase of solubility in milk. Based on the binding data and phenytoin's solubility in buffer, the predicted milk solubility, PMS, in the low fat milk at 37° is:

$$\begin{aligned} \text{PMS} &= \text{solubility in buffer} + \text{solubility in buffer} \times (\text{fraction bound}) \\ &= 40{\cdot}1 + 40{\cdot}1\,(0{\cdot}523) = 61{\cdot}1\ \mu\text{g ml}^{-1} \end{aligned}$$

This value is only 16 per cent of that found experimentally (378·1 ± 17·2). The remarkably higher solubility of sodium phenytoin in milk than in buffer at all temperatures studied is in accord with previous studies where high solubilities in milk, not accounted for solely by drug binding to milk components, were observed for sparingly water-soluble drugs.[14,18] However, as shown in Figure 1, the relative increase in solubility in milk becomes less as temperature increases. This correlates well with the binding data and suggests that the small increase of solubility from 25 to 37° reflects the reduced binding (Table 1). Accordingly, the statistically unchanged binding at 15 and 25° results in a steeper increase in the solubility of phenytoin. The characteristically small effect of fat content on both binding (less than 12 per cent) and solubility (practically nothing) in milk indicates that the amount of fat is an unimportant factor for both binding and solubility of phenytoin in milk. *In vivo* studies have shown that high fat meals do not affect the absorption of phenytoin[19] while co-administration of a high protein meal delays and may reduce the extent of absorption.[19,20] Milk, specifically, has been shown not to affect the absorption of phenytoin.[1] Accordingly, the *in vivo* experiments were performed with low fat milk.

The results from the two control formulations (Table 2, last two columns) confirm that milk does not affect the absorption of phenytoin when it is administered in a solid dosage form. This is in contrast with the *in vitro* data where a 10-fold increase in its solubility in milk was observed. The apparent discrepancy could be attributed to the ability of strong sodium cations to buffer the micro-environment of the solid particles, regardless of the nature of the bulk solution (aqueous or milk). The pH in the micro-environment is elevated and thus the dissolution of the drug, which is a weak acid, is facilitated.

The current study indicates that the freeze-dried formulation significantly increases the rate of absorption and decreases the time to peak serum concentration. It is also worth mentioning that the high intra-subject variation of the AUC when the control formulation was administered with water (Table 2) could be attributed to inconsistent gastric emptying between subjects due to the fasting conditions.[21] As far as the gastric emptying is concerned, aggregation or adhesion of drug particles to stomach mucus[5] could result in differences in the rate of emptying between the water or milk and the undissolved drug substance. It seems reasonable, therefore, that both dissolution and gastric

emptying should be considered as factors that may affect the oral bioavailability of phenytoin. In addition, it has been reported that co-perfusion of nutrient solutions with iso-osmotic phenytoin solution in rats resulted in higher phenytoin permeabilities.[5] Consequently, differences in t_{max} and k_a between the water control formulation and the freeze-dried formulation should be attributed to differences in the dissolution process, gastric emptying and/or intestinal uptake. On the other hand, comparison between the freeze-dried and the milk control formulations indicates that any difference in the absorption process should be attributed to dissolution and/or gastric emptying of drug particles.

In conclusion, it seems that, in contrast to the conclusions suggested by the *in vitro* data, dissolution characteristics *in vivo* remain relatively unchanged by the presence of milk. However, dissolution, at least in part, is still of great importance in controlling the absorption of phenytoin, as indicated by the significant differences in the pharmacokinetic parameters k_a and t_{max} between the control milk formulation and the freeze dried formulation (the administration of which bypasses dissolution). It should be noted, however, that although single dose studies are usually recommended to assess bioavailability,[22] in the case of dose dependent kinetics, the results could be different at steady state.[23] The clinical impact of absorption variability for phenytoin may be moderated since metabolism, which is saturable within the dose-range of phenytoin, may mask absorption variability.

Incorporation of the drug substance into milk and freeze drying of the resulting solution have been shown to greatly improve both the rate and the extent of the bioavailability of sparingly water soluble drugs which are weak acids.[6,7] However, the absence of any change in the extent of bioavailability in the case of sodium phenytoin clearly shows that low water-solubility is not the only factor governing the oral absorption from a drug–milk freeze-dried formulation.

ACKNOWLEDGEMENTS

This work was in part supported by the Ministry of Industry, Energy and Technology, General Secretariate of Research and Technology of Greece. We express our sincere thanks to Dr G. Valsami for valuable discussions and Dr L. Poulos for his excellent supervision of the *in vivo* studies.

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