Rapid In Vitro Conversion of Fosphenytoin Into Phenytoin in Sera of Patients With Liver Disease: Role of Alkaline Phosphatase

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Fosphenytoin, a phosphate ester pro drug of phenytoin, also cross-reacts with the fluorescence polarization immunoassay (FPIA) for phenytoin. We measured fosphenytoin concentrations using the FPIA kit and TDx analyzer. We prepared serum pools from normal volunteers and patients with liver disease. None of them received either fosphenytoin or phenytoin. Fosphenytoin standard solution (1 mg/ml) was prepared in water. We supplemented aliquots of normal and liver pools with known amounts of fosphenytoin and measured the concentrations at different time intervals. The conversion of fosphenytoin to phenytoin was slow in sera with normal alkaline phosphatase activities. The conversion was rapid in sera collected from patients with liver disease who also had high alkaline phosphatase activities. The observed concentrations were close to target concentrations within 0–2 min of supplementation with fosphenytoin. Surprisingly, the observed concentration then started to decline slightly but significantly with longer incubation time. In contrast, the observed

concentration increased steadily in serum with normal alkaline phosphatase activity. For example, in the normal pool supplemented with 15.0 $\mu\text{g/ml}$ fosphenytoin (as the phenytoin equivalent), the observed concentrations were 6.9, 7.3, 7.7, 8.3, and 9.8 $\mu\text{g/ml}$ at 0–2, 10, 20, 30, and 60 min, respectively. However, in a serum pool prepared from patients with liver disease and supplemented with 15.0 $\mu\text{g/ml}$ of fosphenytoin (alkaline phosphatase: 2547 U/l), the observed phenytoin concentrations were 12.9, 12.1, 11.0, 10.7, and 10.7 $\mu\text{g/ml}$ at 0–2, 10, 20, 30, and 60 min, respectively. When we added alkaline phosphatase to the normal serum pool, we observed rapid conversion of fosphenytoin into phenytoin within 10 min, but the concentrations then declined with longer incubation time. However, when we repeated the experiment with protein-free ultrafiltrate, we observed rapid conversion of fosphenytoin to phenytoin, but the concentration did not decline with longer incubation time. *J. Clin. Lab. Anal.* 15:244–250, 2001. © 2001 Wiley-Liss, Inc.

Key words: fosphenytoin; phenytoin; liver disease; alkaline phosphatase

INTRODUCTION

Fosphenytoin is a phosphate ester pro drug of phenytoin. The chemical name is 5,5-diphenyl-3-[(phosphoxy)methyl]-2,4-imidazolidinedione. Fosphenytoin was designed to overcome many shortcomings associated with parenteral administration of phenytoin. Because of phenytoin's limited solubility in water, phenytoin sodium USP 50 mg/ml consists of an aqueous vehicle of 40% propylene glycol and 10% alcohol at pH 12. This preparation has many limitations. Intravenous administration can be painful and irritating to the vein (1–3). Rapid intravenous injection can cause cardiovascular collapse (4,5). Intramuscular administration is extremely painful due to crystallization of phenytoin at the injection site due to lower pH of tissue (6). Fosphenytoin is readily soluble in water and can be administered safely by intravenous or intramuscular routes. Fosphenytoin has no known pharmacologi-

cal activity, and fosphenytoin dosing uses a phenytoin equivalent to minimize dosing error (7). The pharmacological activity of fosphenytoin is due to its active metabolite, phenytoin. For each 1 mmol of fosphenytoin administered, 1 mmol of phenytoin is generated. However, hydrolysis of fosphenytoin also generates two other metabolites: phosphate and formaldehyde. The concentration of formaldehyde generated is very low and has no adverse pharmacological effect. The mecha-

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nism of hydrolysis of fosphenytoin into phenytoin *in vivo* is not completely understood, but phosphatase enzymes play an important role.

Fosphenytoin is strongly bound to serum proteins (90–95%), primarily to albumin. Binding of fosphenytoin is concentration dependent (8). Fosphenytoin displaces phenytoin from a protein binding site (9). Carbamazepine, diazepam, or phenytoin have no effect on the binding of fosphenytoin to serum protein, but phenylbutazone and sulfisoxazole result in significant increases in free fraction of fosphenytoin (10). Fosphenytoin has not been detected in urine. No drug is known to affect the metabolism of fosphenytoin to phenytoin.

The clearance of fosphenytoin to phenytoin is increased in sera of patients with liver disease, but the clearance of the active metabolite phenytoin is not increased. Therefore, special precaution should be taken when fosphenytoin is prescribed to this patient population (Fosphenytoin package insert, Parke-Davis, Morris Plains, NJ). We suspected that elevated concentrations of liver enzyme might play an important role in rapid conversion of fosphenytoin to phenytoin. We used an *in vitro* model for this study and used the fluorescence polarization immunoassay (FPIA) to measure fosphenytoin concentrations as phenytoin equivalents, taking advantage of the cross-reactivity of fosphenytoin

TABLE 1. Concentrations of albumin, bilirubin, ALT, AST, and ALP in normal and liver pools

Specimen	Albumin (gm/dL)	Bilirubin (mg/dL)	ALT (U/L)	AST (U/L)	ALP (U/L)
Normal pool 1	3.9	0.4	16	18	85
Liver pool 1	2.9	2.1	172	293	174
Liver pool 2	3.6	5.9	122	50	2,547
Liver pool 3	2.7	5.9	427	386	490

with the phenytoin immunoassay. We observed rapid conversion of fosphenytoin into phenytoin as evidenced by increased phenytoin concentration. Surprisingly, the phenytoin concentrations then started to decline steadily, indicating the possibility of reestablishing a thermodynamic equilibrium.

MATERIALS AND METHODS

Fosphenytoin was purchased from Parke-Davis. Alkaline phosphatase (ALP), type VII from bovine intestinal mucosa in alkaline solution in 3.0 M sodium chloride containing 1 mM magnesium chloride, 0.1 mM zinc chloride, and 30 mM triethanolamine at pH 7.6 was obtained from Sigma Chemical Company (Catalog #P6774, St. Louis, MO). Fosphenytoin

Fig. 1. Target and observed apparent phenytoin concentrations (measured by FPIA) observed in specimens supplemented with various amounts of fosphenytoin.

and phenytoin concentrations in serum were measured by FPIA for phenytoin (Abbott Laboratories, Abbott Park, IL). Concentrations of ALT, AST, ALP, and bilirubin were measured by using a Hitachi 747 automated analyzer.

We prepared one serum pool from normal volunteers, and three different serum pools from patients with liver disease (Liver 1, Liver 2, and Liver 3). We routinely received serum samples from patients with liver disease in our clinical laboratory for various tests. Serum pools were prepared from leftover specimens after performing and reporting all tests ordered by the clinicians. These leftover specimens would have been discarded otherwise.

In the first experiment, we studied cross-reactivity of fosphenytoin with the FPIA assay for phenytoin. For this purpose aliquots of drug-free serum were supplemented with various concentrations of fosphenytoin, and apparent phenytoin activities were measured using the FPIA.

In another experiment, we supplemented normal and serum pools prepared from patients with liver disease with two different concentrations of fosphenytoin (15.0 and 25.0 $\mu\text{g}/\text{ml}$ of phenytoin equivalent) to represent a therapeutic and a toxic situation. Fosphenytoin concentrations were measured at 0–2 min (immediately after incubation), and at 10-, 20-, 30-, and 60-min intervals. The observed phenytoin concen-

trations reflected the combined concentration of unconverted fosphenytoin and phenytoin (derived by in vitro hydrolysis of fosphenytoin). A close match between the target concentration and observed concentration indicated almost complete in vitro conversion of fosphenytoin into phenytoin because fosphenytoin showed lower cross-reactivity with the FPIA.

In a separate experiment, we used another drug-free serum pool containing a normal concentration of ALP to investigate the effect of adding endogenous ALP to the conversion of fosphenytoin into phenytoin. We also prepared a protein-free ultrafiltrate of the same serum pool by centrifuging a specimen with the Centrifree Micropartition System (Amicon, Danver, MA) for 30 min at 1,500 g. The Centrifree ultrafiltration device contains a membrane with a molecular cutoff of 30,000 Dalton. Then we supplemented two aliquots of the normal pool with a known concentration of fosphenytoin. We also supplemented two aliquots of ultrafiltrate with the same concentration of fosphenytoin. Then fosphenytoin concentrations in all four specimens were measured using the FPIA, and the values were expressed as 0 min. Then 10 μl of ALP solution was added to one aliquot of serum and one aliquot of ultrafiltrate already supplemented with fosphenytoin. Another aliquot was treated as a control. Again, we measured phenytoin concentrations at 10-, 20-, 30-, and 60-min intervals.

Fig. 2. In vitro conversion of fosphenytoin into phenytoin in normal pools and serum pools prepared from patients with liver disease (Liver 1, Liver 2, and Liver 3). All pools were supplemented with 15.0 $\mu\text{g}/\text{ml}$ of fosphenytoin

(phenytoin equivalent). FPIA was used for measuring apparent phenytoin concentrations.

Fig. 3. In vitro conversion of fosphenytoin into phenytoin in a normal pool and serum pools prepared from patients with liver disease (Liver 1, Liver 2, and Liver 3). All pools were supplemented with 25.0 $\mu\text{g/ml}$ of

fosphenytoin (phenytoin equivalent). FPIA was used for measuring apparent phenytoin concentrations.

In another experiment, we incubated 1-ml aliquots of drug-free serum with 10 and 25 μl of ALP for 1 hr. Then we further supplemented the aliquots with fosphenytoin (target concentration 15.0 $\mu\text{g/ml}$ phenytoin equivalent) and measured apparent phenytoin concentrations at 10-, 20-, 30-, and 60-min intervals.

To study the effect of ALP on phenytoin concentrations as measured by the FPIA, we prepared two additional serum pools from patients receiving phenytoin. To 1-ml aliquots of each serum pool, we added 10 μl of ALP solution. Again, serum phenytoin concentrations were measured at regular time intervals.

Statistical analyses were performed using an independent two-tailed *t*-test. We considered a difference statistically significant only at a 95% or higher confidence interval ($P < 0.05$).

RESULTS AND DISCUSSION

The concentrations of albumin, ALT, AST, ALP, and bilirubin in the normal serum pool and serum pools prepared from patients with liver disease are given in Table 1. As expected, concentrations of bilirubin were significantly elevated in serum pools prepared from patients with liver disease. Moreover, albumin concentrations were lower in liver pools

compared to the normal pool. We deliberately prepared one serum pool containing a very high concentration of ALP and another pool with a much lower concentration of ALP. However, both pools had the same concentrations of bilirubin. The purpose was to study the effect of alkaline phosphatase on in vitro hydrolysis of fosphenytoin into phenytoin.

Fosphenytoin showed lower cross-reactivity with the FPIA of phenytoin. The cross-reactivity was not linear, and decreased with increased concentration of fosphenytoin. In sample 1, the target fosphenytoin concentration as phenytoin equivalent was 5.0 $\mu\text{g/ml}$. The observed value was 4.2 $\mu\text{g/ml}$ (84% cross-reactivity). However, in sample 2, the target and observed concentrations were 10.0 $\mu\text{g/ml}$ and 7.7 $\mu\text{g/ml}$, respectively, indicating a 77% cross-reactivity. The cross-reactivity dropped to 52.5% in sample 5, in which the observed concentration was 21.0 $\mu\text{g/ml}$ (target concentration: 40 $\mu\text{g/ml}$). The results are presented in Fig. 1.

The in vitro hydrolysis of fosphenytoin into phenytoin was slow in the serum pool containing a normal concentration of alkaline phosphatase. For example, in the normal pool containing 85 U/L of ALP and supplemented with 15.0 $\mu\text{g/ml}$ of fosphenytoin (in phenytoin equivalent), the observed fosphenytoin concentration was 6.9 $\mu\text{g/ml}$ phenytoin equivalent at 0–2 min. The concentration was increased to 9.8 $\mu\text{g/}$

Fig. 4. Effect of bovine intestinal mucosal ALP on in vitro hydrolysis of fosphenytoin into phenytoin in the normal serum pool and protein-free ultrafiltrate prepared from the normal serum pool.

ml after 60 min of incubation. The increase in observed concentration was due to *in vitro* hydrolysis of fosphenytoin into phenytoin. In the serum pool prepared from patients with liver disease (Liver 1) containing 174 U/L of ALP, the initial concentration was 6.8 µg/ml. The observed concentration then increased to 9.9 µg/ml after 10 min of incubation. The observed concentration was 13.6 µg/ml after 60 min of incubation, indicating that almost all fosphenytoin was hydrolyzed to phenytoin. The pool was supplemented with 15.0 µg/ml phenytoin equivalent of fosphenytoin. Therefore, complete hydrolysis of fosphenytoin should produce an observed concentration of 15.0 µg/ml. In the second serum pool (Liver 2), the observed concentration within 0–2 min of incubation was 12.9 mg/ml, indicating almost instantaneous hydrolysis of fosphenytoin into phenytoin due to a high ALP concentration of 2,547 U/L. Surprisingly, the observed concentration decreased slightly with longer incubation time. The observed concentrations at 10-, 20-, 30-, and 60-min intervals were 12.1 µg/ml, 11.0 µg/ml, 10.7 µg/ml, and 10.7 µg/ml, respectively (Fig. 2). When we supplemented other aliquots of the normal pool and serum pools prepared from patients with liver disease with 25.0 µg/ml of fosphenytoin, we observed similar results (Fig. 3).

We observed no correlation between bilirubin concentrations and the rapidness of hydrolysis of fosphenytoin into phenytoin. For example, the second and third serum pools prepared from patients with liver disease (Liver 2 and 3) had the same concentrations of bilirubin (5.9 mg/dl). However, ALP concentrations were very different. We observed much faster hydrolysis of fosphenytoin into phenytoin in Liver 2 because it had a higher amount of ALP than Liver 3. Again, ALT and AST did not play any role in the hydrolysis of fosphenytoin into phenytoin.

To further investigate the role of ALP in hydrolysis of fosphenytoin into phenytoin, we supplemented a normal serum pool containing 90 U/L of ALP with 15.0 µg/ml phenytoin equivalent of fosphenytoin. We also supplemented the protein-free ultrafiltrate prepared from the same serum pool with the same concentration of fosphenytoin. The ultrafiltrate did not show any ALP activity. Then we put 1 ml of supplemented serum in one test tube and marked it as "control," and put another 1 ml in another test tube and marked it as "ALP." We repeated the same process with the ultrafiltrate supplemented with fosphenytoin. Then the fosphenytoin concentrations were measured in all specimens. Next, 10 µl ALP solution was added to the serum in the "ALP" tube as well as to the "ALP" ultrafiltrate in order to achieve a very high ALP. Then we measured phenytoin concentrations at 10-, 20-, 30-, and 60-min intervals (Fig. 4). The concentration in the control serum specimen increased from 7.4 µg/ml (0 min) to 8.9 µg/ml after 10 min of incubation, showing a little hydrolysis of fosphenytoin into phenytoin. However, the serum specimen containing fosphenytoin supplemented with ALP showed a fosphenytoin concentration of 14.9 µg/ml after

10 min of incubation, indicating complete hydrolysis of fosphenytoin to phenytoin. The observed phenytoin concentration was also observed in the ultrafiltrate, as expected. However, with longer incubation, the observed phenytoin concentration in the serum supplemented with ALP declined slightly. In contrast, we observed a slight increase in observed phenytoin concentration in the ultrafiltrate supplemented with ALP.

When drug-free serum was supplemented with ALP prior to addition of fosphenytoin, we observed a rapid conversion of fosphenytoin into phenytoin followed by a slight decrease in observed phenytoin concentration, similar to that observed with the serum pool supplemented first with fosphenytoin and then ALP. Therefore, ALP does not alter serum protein binding of phenytoin or fosphenytoin, which could have affected the FPIA results. Moreover, when serum pools prepared from patients receiving phenytoin were incubated with ALP for 60 min, we observed no statistically significant change in phenytoin concentrations, indicating that ALP can not modify phenytoin concentration as measured by the FPIA.

Rapid hydrolysis of fosphenytoin into phenytoin by ALP followed by slight but significant decreases in observed concentration is interesting. Stapp et al. (11) showed that conversion of fosphenytoin into phenytoin is relatively slow. Our observation is in agreement with theirs. Cao et al. (12) also demonstrated recently that ALP from bovine intestinal mucosa enhances the conversion of fosphenytoin into phenytoin *in vitro*. They also showed that in the presence of 33 U/ml (33,000 U/L) of ALP, complete hydrolysis was achieved in 5 min at 37°C. Again, our observation is in agreement with theirs. Cao et al. (12) did not report any effect of long-term incubation on the observed phenytoin concentrations. We observed a rapid increase in phenytoin concentration, indicating complete hydrolysis followed by slight but significant decreases in observed phenytoin concentration. However, such an effect is absent in the protein-free ultrafiltrate, indicating that serum proteins may play a role in this observation.

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