Reconversion of Fosphenytoin in the Presence of Intestinal Alkaline Phosphatase

Christopher N. TenHoor[1] and Barbra H. Stewart[1,2]

Received June 14, 1995; accepted
KEY WORDS: fosphenytoin; prodrugs; phosphate monoesters; intestinal alkaline phosphatase; targeted reconversion.

INTRODUCTION

The gastrointestinal absorption of poorly water soluble drugs can often limit oral bioavailability and therapeutic benefit. One solution is modification of the drug molecule through chemical derivatization to a prodrug which will then be reconverted to the active moiety in the body.

One prodrug strategy which has shown promise involves making water-soluble derivatives which are substrates for enzymes in the brush border membrane region of intestinal mucosal cells. This membrane metabolism or targeted prodrug strategy results in reconversion of prodrug to the lipophilic parent drug in high concentration adjacent to the intestinal membrane, resulting in an increased driving force for absorption (1). Early work on the 21-phosphate esters of hydrocortisone and prednisolone showed that these compounds demonstrated somewhat higher plasma levels when compared to their respective parent compounds after oral administration to beagle dogs (2). In addition, rat intestinal ring studies verified that both the hydrocortisone and prednisolone prodrugs underwent hydrolysis prior to absorption by the intestinal mucosa (1,3). Doyle and Vyas (4) found that the 4'-phosphate ester of etoposide (etopofos) exhibited both improved pharmaceutical properties and equivalent *in vivo* antitumor activity to etoposide in a variety of murine models.

Fosphenytoin, the sodium mono-phosphate ester derivative of phenytoin (5,5,-diphenylhydantoin), is a model compound for the application of the targeted prodrug approach to oral drug delivery. The addition of the phosphate ester functionality resulted in over a 7,000-fold increase in aqueous solubility from 0.02 mg/mL to 142 mg/mL (5). Fosphenytoin has also been shown to be stable in aqueous solutions. In addition, phenytoin, with a pKa of 8.1, a log membrane-water partition coefficient of 2.4, and a dimensionless membrane permeability ≥ 4 (6), provides the desired membrane transport characteristics for the parent drug.

The present study quantitated the activity and determined the kinetic parameters of alkaline phosphatase (orthophosphoric monoester phosphohydrolase, E.C. 3.1.3.1) in a variety of preparations from both the jejunum and colon. In addition, the stability of fosphenytoin and its rate of reconversion to phenytoin was determined in the presence of the above-mentioned preparations as well as purified alkaline phosphatase from a commercial source. Finally, inhibition studies were conducted to confirm the involvement of alkaline phosphatase in the reconversion of fosphenytoin.

MATERIALS AND METHODS

Materials

Fosphenytoin (PD 135711, lot 7/V) and phenytoin (PD 005674, lot 16/E) were both obtained on-site from the Department of Chemical and Biological Information (Parke-Davis Pharmaceutical Research, Ann Arbor, MI). All other chemicals were reagent grade or better. Trichloroacetic acid (TCA), monobasic ammonium phosphate, monobasic potassium phosphate, dibasic sodium phosphate, calcium chloride, and sodium chloride were purchased from EM Science (Darinstadt, Germany). 4-Nitrophenylphosphate (4NPP), 4-nitrophenol (4NP), bis-Tris propane-HCl, and zinc chloride were obtained from Aldrich Chemical Co. (Milwaukee, WI). D-mannitol, trizma base (Tris[hydroxymethyl]aminomethane), MES (2-[N-Morpholino]ethanesulfonic acid), HEPES (N-[2-Hydroxyethyl]piperazine-N'-[2-ethanesulfonic acid]), Folin & Ciocalteu's Phenol Reagent, Biuret Reagent, Bovine Serum Albumin protein standards, and the commercial purified alkaline phosphatase ($AlkPase/_{Comm}$) were purchased from Sigma Chemical Co. (St. Louis, MO). Methanol and potassium chloride were purchased from Mallinckrodt (Paris, KY). Magnesium chloride was obtained from the J.T. Baker Co. (Phillipsburg, NJ). Water used in all studies was from a Millipore MILLI Q water system (Bedford, MA).

Apparatus

All pH determinations were made using a Φ 40 pH Meter (Beckman Instruments Inc., Fullerton, CA). The high performance liquid chromatography (HPLC) assay used in the analysis of both fosphenytoin and phenytoin consisted of SP 8780 Autosampler (Spectraphysics, San Jose, CA) with a 100 μL loop. The mobile phase, 50 mM ammonium phosphate (pH 6.5):methanol (65:35 v/v), was delivered at 0.8 mL/min via a SP 8700XR pump (Spectraphysics, San Jose, CA). Both the guard column, a 3.2 mm x 30 mm Hypersil C18 (5 μm resin), and column, a 3.2 mm x 250 mm Hypersil C18 (5 μm resin) were maintained at 40°C by Model III Temperature Control Unit (Rainin Instrument Co., Woburn, MA). The eluent was monitored at 214 nm by a Spectroflow 783 Absorbance Detector (Kratos Analytical Instruments, Ramsey, NJ) and integrated by a SP 4270 Integrator (Spectraphysics, San Jose, CA). The quantitation of the alkaline phosphatase activity was performed using a DU 70 Spectrophotometer (Beckman Instruments Inc., Fullerton, CA).

Preparation of Purified Membrane Fractions

The membrane purification was based on the Ca^{2+} precipitation procedure outlined by Kessler *et al.* (7). Fasted,

[1] Department of Pharmacokinetics and Drug Metabolism, Parke-Davis Research Division, Warner-Lambert Co., Ann Arbor, Michigan 48105.
[2] To whom correspondence should be addressed.

0724-8741/95/1100-1806$07.50/0

male Wistar rats, 250-350 g, were used as the animal source. Membranes were purified from both the jejunum and colon. The jejunum was identified by first locating the ligament of Treitz and then excising approximately 30 cm of intestine distal to that marker. The colon was defined as the length of large intestine from the cecum to the rectum. The intestinal mucosa from each segment was scraped, suspended in buffer (50 mM mannitol and 2 mM Tris, adjusted to pH 7.1 with 2 mM HEPES), and homogenized using a blender for 5 minutes. Aliquots of this crude homogenate from both the jejunum and colon were saved to calculate the enrichment factor of the final purified membranes and for use in reconversion studies. To the remaining crude fraction, 1 M $CaCl_2$ was added to a final concentration of 10 mM and centrifuged at 3000 g for 15 minutes at 0°C. The supernatant was collected and centrifuged at 27,000 g for 30 minutes at 0°C to obtain pellets. The pellets were collected and resuspended in buffer (100 mM KCl, 100 mM mannitol, and 10 mM Tris, adjusted to pH 7.4 with 2 M HEPES), and centrifuged again at 27,000 g for 30 minutes at 0°C to obtain the purified membranes (BBM: brush border membranes, PCM: purified colonic membranes).

Perfusate Generation

The jejunum or colon of 250-300 g fasted, male Wistar rats was identified as outlined above. A proximal glass cannula was inserted and connected to a Harvard withdrawal/infusion pump (Harvard Apparatus, South Natick, MA). A second cannula was positioned approximately 15 cm distal to the first cannula in the jejunum and approximately 9 cm distal in the colon and led to a collection tube. Buffer containing 135 mM NaCl, 10 mM MES, and 5 mM KCl (pH 7.4) was oscillated at 30 mL/min through the respective segment for a total of 90 minutes.

Protein Quantitation

The quantitation of the protein content in all alkaline phosphatase-containing preparations was based on the methodology reported by Ohnishi and Barr (8).

Alkaline Phosphatase Activity

The quantitation of alkaline phosphatase activity was based on the methodology reported by Gilles-Baillien and Croux (9). Activity was measured in 2 mL of medium which contained 5 mM $MgCl_2$, 0.25 mM $CaCl_2$, 0.1 mM $ZnCl_2$, 50 mM bis-Tris-propane-HCl, and 5 mM 4NPP (substrate). Each reaction was carried out at pH 9.5 or 7.4, and 37°C. After addition of the enzyme-containing preparation, the reaction was followed for 5 minutes. Alkaline phosphatase activity was calculated from the change in absorbance at 405 nm, representing the amount of 4-nitrophenol released, and expressed as picomoles 4NPP/second/mL (pkat/mL).

Reconversion Studies

Aliquots of fosphenytoin (100 μL) were mixed with an equal volume of the appropriate alkaline phosphatase-containing preparation in individual Eppendorf tubes. The tubes were placed in a reciprocating water bath and shaken at 60 cycles/minute. All incubations were performed at 37°C. The conversion was quenched by addition of 50 μL 20% TCA. Blanks representing zero time points were prepared by incubating fosphenytoin with quenched enzyme preparations. The tubes were then vortexed and centrifuged at 14,000 r.p.m. for 12 minutes (Eppendorf 5415C Centrifuge, Brinckman Instruments Inc., Westbury, NY). The resulting supernatant was analyzed by HPLC. The concentrations of fosphenytoin and phenytoin were back-calculated from standard curves consisting of external standards of both compounds. Reconversion rates were calculated from the slopes of the initial linear portion of the concentration versus time plots. The simple linear regression analyses were performed using Cricket Graph Macintosh software (Cricket Software, Malvern, PA).

RESULTS AND DISCUSSION

Alkaline Phosphatase Activity

The alkaline phosphatase activities for the AlkPase/$_{Comm}$ and jejunal preparations are shown in Figure 1. For each of the enzyme sources, the protein concentration was normalized to approximately 4.4 mg/mL while the concentration of 4NPP was varied from 1 μM to 5000 μM. The calculated K_m values for the AlkPase/$_{Comm}$, crude jejunal fraction, and BBM, were 3.3-, 33.9-, and 18.0 μM respectively. The V_{max} values for these same preparations were 1,010-, 15,310-, and 215,260 pmoles/sec/mL. Based on these numbers, the first-order rate constants (V_{max}/K_m) for both the AlkPase/$_{Comm}$ and crude fraction were approximately 30 times lower than that calculated for the BBM.

Based on the kinetics and first-order rate constants for the hydrolysis of 4NPP by the jejunal preparations, the brush border membranes have the greatest enzymatic activity. Twelve- to fifteen-fold increases in alkaline phosphatase activity between the crude and purified intestinal fractions have been reported (7, 10). On a pkat/mg protein basis, our preparations showed a 15-fold enrichment between the jejunal crude homogenate and BBMs and a 21-fold enrichment for the colonic fractions. In addition, the 38-fold increase in hydrolysis rate with BBMs compared to the AlkPase/$_{Comm}$ validates the use of BBMs as a model enzyme source for examining reconversion of prodrugs targeted for intestinal alkaline phosphatase.

Reconversion Studies

The results for the reconversion of fosphenytoin are shown in Table I. Perfusate was used to stimulate conditions encountered in the lumen of the gastrointestinal tract, while the purified preparations represented alkaline phosphatase levels at the brush border membrane. With the jejunal preparations, the half-life decreased 6-fold between the perfusate and crude homogenate. In the presence of the purified BBM, the reconversion to parent phenytoin was almost immediate. Fosphenytoin was essentially stable in the colonic perfusate, while the alkaline phosphatase activity in the crude colon homogenate resulted in a half-life of just over 10 minutes. A

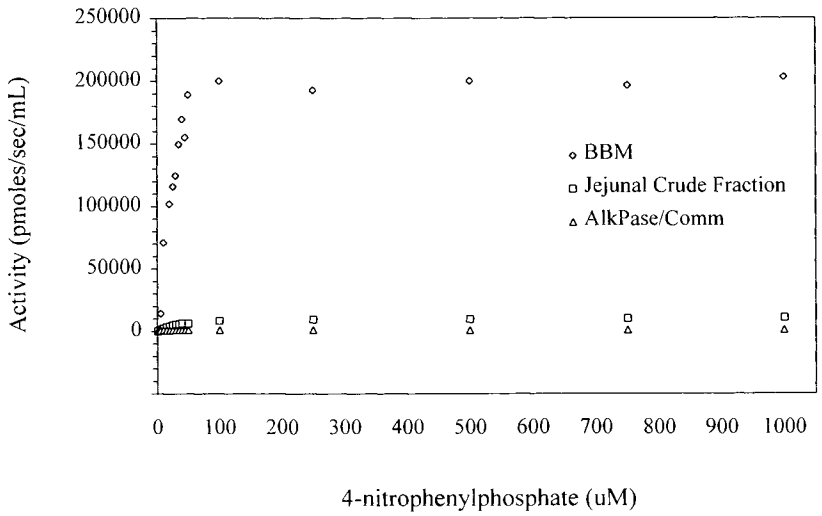

Fig. 1. Alkaline phosphatase activity as a function of 4-nitrophenylphosphate concentration at pH 7.4. Each point represents one determination. Crude fraction and BBM were prepared from pooled mucosal scrapings (n = 8 rats).

further increase in activity resulted in a 3-fold decrease in half-life in the PCMs. Although direct extrapolation from *in vitro* findings to the *in vivo* situation is not possible, the results indicate a rapid conversion of fosphenytoin to phenytoin in the small intestine.

According to McComb *et al.* (11), alkaline phosphatase activity in the intestinal wall is higher than in other tissues. An ileal mucosa to colonic mucosa activity ratio ranging from 4:1 to 11:1 in tissues from a variety of mammalian species was reported. Lev and Griffiths (12) and Hirano *et al.* (13) reported human small intestine to colon alkaline phosphatase activities of 8:1 and 16:1, respectively. As Table I shows, the jejunal and colonic preparations used in the reconversion studies follow this same trend, although the magnitude of the difference in activity between the two enzymes was greater than that seen in the referenced studies. The increase in activity (pkat/mg protein) between the jejunal and colonic preparations for the crude fraction and purified membranes was 114- and 82-fold, respectively. Based on the activity of alkaline phosphatase isolated from the jejunal brush border membranes of the rat, the subsequent reconversion rate of the prodrug in the presence of these enzymes, and the presence of significant quantities of alkaline phosphatase in the human intestine, the targeted prodrug approach to oral dosing appears to be a valid drug delivery strategy for fosphenytoin.

Table I. Reconversion of Fosphenytoin in the Presence of Intestinal Phosphatases at pH 7.4[a]

Enzyme source	Protein (mg/mL)	Activity[c] (pkat/mL)	Fosphenytoin half-life (seconds)
Jejunal perfusate	n.d.[b]	25,230	476 (±110)
Crude jejunum	4.1 (±0.9)	96,620	79 (±9)
BBM[d]	12.2 (±2.0)	4,368,900	1.2 (±0.1)
Colonic perfusate	n.d.	320	stable[e]
Crude colon	3.8 (±0.5)	790	606 (±132)
PCM	2.7 (±0.6)	11,980	186 (±33)

[a] data reported as mean ± standard deviation, n ≥ 3.
[b] not determined.
[c] measured at pH 9.5, based on hydrolysis of 4NPP.
[d] extrapolated values, assuming linearity.
[e] no detectable degradation (n = 6 of 8), $t_{1/2}$ 1204–2921 seconds (n = 2).

Inhibition Studies

In order to verify that both 4NPP and fosphenytoin were undergoing reconversion by the same enzyme system, inhibition studies were conducted using inorganic phosphate in addition to 4NPP and fosphenytoin. The results for the inhibition studies are shown in Table II. For each of the inhibition experiments, commercially available alkaline phosphatase (AlkPase/$_{Comm}$), normalized to 4 mg/mL, was used. In the first set of experiments, 4NPP, at a concentration equal to the K_m (3.3 μM), was incubated with enzyme containing inorganic phosphate or fosphenytoin as potential in-

Table II. Inhibition of Alkaline Phosphatase (AlkPase/$_{Comm}$) in the presence of Inorganic Phosphate, 4NPP, and Fosphenytoin at pH 7.4[a]

Sample	Concentration (mM)	Velocity (nmoles 4NP/sec)
Control (4NPP)	0.003	0.034 (±0.003)
+Pi[b]	67	n.d.[c]
+Fos	67	n.d.
Control (fosphenytoin)	0.033	116.80 (±7.07)
+Pi	67	n.d.
+4NPP	67	n.d.

[a] data reported as mean ± standard deviation, n = 3. All solutions incubated with AlkPase/$_{Comm}$ (4 mg/mL).
[b] represents combination of KH_2PO_4 (13 mM) and Na_2HPO_4 (54 mM).
[c] no 4NP detected.

hibitors. A Sorensen buffer (pH 7.4) was used with total phosphate equal to 67 mM. In the absence of inhibitor, 4NP formed at a rate of 0.03 nmoles/second. However, in the presence of phosphate or fosphenytoin, the reaction was completely inhibited. In the second set of experiments, fosphenytoin (33 μM) was incubated with the alkaline phosphatase in the presence or absence of either phosphate or 4NPP. While fosphenytoin was more rapidly converted than 4NPP in the absence of inhibitors, the presence of either phosphate or 4NPP completely abolished the formation of phenytoin.

The cross-inhibition observed between fosphenytoin and 4NPP was consistent with both compounds being substrates for the same enzyme system. In addition, the inhibition of the reaction by inorganic phosphate supports alkaline phosphatase as the operative enzyme, as opposed to reconversion by non-specific esterases. Inorganic phosphate is a classic inhibitor of alkaline phosphatase and forms tight bonds with all alkaline phosphatases investigated including the intestinal form (14, 15).

CONCLUSIONS

Based on the kinetic analysis of the cleavage of 4NPP and the inhibition by inorganic phosphate, the purified brush border membrane preparations provided an optimal source of active alkaline phosphatases for use in screening potential candidates. The results also provide information which may be useful in the design of other prodrug candidates. Our results demonstrated that fosphenytoin was cleaved more rapidly than 4NPP. We believe this to be due to the configuration of the phosphate ester and its accessibility to the active site of the enzyme. This demonstrates the importance of molecular design to the success of the targeted prodrug strategy by conferring reconversion specificity. Lastly, we have shown that fosphenytoin is indeed a compound that could make use of this delivery strategy. Both the prodrug and parent compounds have the desired physicochemical properties; furthermore the reconversion rate, upon exposure to the purified jejunal membranes, is rapid and should provide high concentrations for an increased driving force for absorption of phenytoin.

REFERENCES

1. G.L. Amidon, B.H. Stewart, and S. Pogany. Improving the intestinal mucosal cell uptake of water insoluble compounds. *J Controlled Release* 2:13-26 (1985).
2. D. Fleisher, K.C. Johnson, B.H. Stewart, and G.L. Amidon. Oral absorption of 21-corticosteroid esters: A function of aqueous stability and intestinal enzyme activity and distribution. *J. Pharm. Sci.* 75:934-939 (1986).
3. B.H. Stewart, G.L. Amidon, and R.K. Barbec. Uptake of prodrugs by rat intestinal mucosal cells: Mechanism and pharmaceutical implications. *J. Pharm. Sci.* 75:940-945 (1986).
4. T.W. Doyle and D.M. Vyas. Second generation analogs of etoposide and mitomycin C. *Cancer Treat. Rev.* 17:127-131 (1990).
5. S.A. Varia, S. Schuller, K.B. Sloan, and V.J. Stella. Phenytoin prodrugs III: Water-soluble prodrugs for oral and/or parenteral use. *J. Pharm. Sci.* 73:1068-1073 (1984).
6. D. Fleisher, N. Sheth, H. Griffin, M. McFadden, and G. Aspacher. Nutrient influences on rat intestinal phenytoin uptake. *Pharm. Res.* 6:332-337 (1989).
7. M. Kessler, O. Acuto, C. Storelli, H. Murer, M. Muller, and G. Semenza. A modified procedure for the rapid preparation of efficiently transporting vesicles from brush border membranes. *Biochim. Biophys. Acta.* 506:136-154 (1978).
8. S.T. Ohnishi and J.K. Barr. A simplified method of quantitating protein using the Biuret and phenol reagents. *Anal. Biochem.* 86:193-200 (1978).
9. M. Gilles-Baillien and E. Croux. Bound and purified alkaline phosphatase from rat duodenal and jejunal brush-border membranes: Kinetics and magnesium stimulation. *Arch. Int. Phys. Biochim. Biophys.* 100:23-26 (1991).
10. A. Tsuji, T. Terasaki, I. Tamai, and H. Hirooka. H+ gradient-dependent and carrier-mediated transport of cefixime, a new cephalosporin antibiotic, across brush-border membrane vesicles from rat small intestine. *J. Pharm. Exp. Ther.* 241:594-601 (1987).
11. R.B. McComb, G.N. Bowers, and S. Posen. *Alkaline Phosphatase,* Plenum Press, New York, 1979.
12. R. Lev and W.C. Griffiths. Colonic and small intestinal alkaline phosphatase. *Gastroenterology* 82:1427-1435 (1982).
13. K. Hirana, H. Matsumoto, T. Tanaka, Y. Hayashi, S. Iino, U. Domar, and T. Stigbrand. Specific assays for human alkaline phosphatase isozymes. *Clin. Chim. Acta.* 166:265-273 (1987).
14. Y. Dupuis, S. Tardivel, Z. Porembska, and P. Fournier. Effect of some alkaline phosphatase inhibitors on intestinal calcium transfer. *Int. J. Biochem.* 23:175-180 (1991).
15. S. Tardivel, L. Razanamaniraka, Z. Porembska, G. Crouzoulon, P. Fournier, and Y. Dupuis. Homodimer and heterodimer forms of adult rat intestinal alkaline phosphatase. *Life Sci.* 43:2059-2065 (1988).