A Novel Prodrug Approach for Tertiary Amines. 2. Physicochemical and in Vitro Enzymatic Evaluation of Selected *N*-Phosphonooxymethyl Prodrugs

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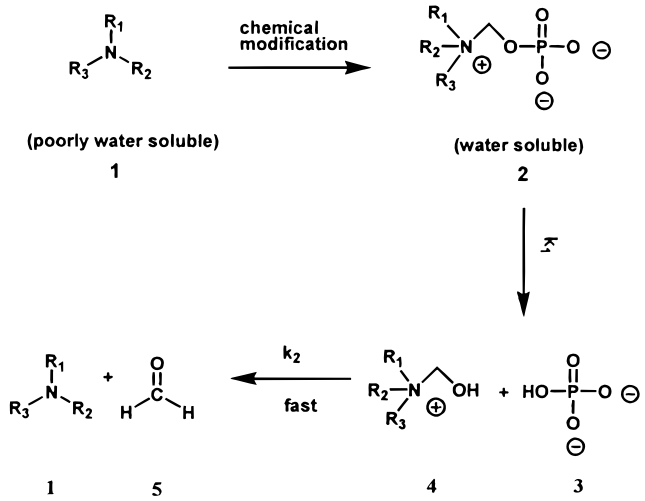
Received September 21, 1998. Final revised manuscript received May 21, 1999.
Accepted for publication May 26, 1999.

Abstract □ Quaternary amine prodrugs resulting from *N*-phosphonooxymethyl derivatization of the tertiary amine functionality of drugs represents a novel approach for improving their water solubility. Separate reports have demonstrated the synthetic feasibility and rapid and quantitative prodrug to parent drug conversion in rats and dogs. This work is a preliminary evaluation of the physicochemical and in vitro enzymatic reversion properties of selected prodrugs. The loxapine prodrug had over a 15 000-fold increase in aqueous solubility relative to loxapine free base at pH 7.4. The loxapine prodrug was also shown to be quite stable at neutral pH values. The time for degradation product (parent drug) precipitation from an aqueous prodrug formulation would be expected to dictate the shelf life. Using this assumption, together with solubility and elevated temperature chemical stability studies, the shelf life of a parenteral formulation of the loxapine prodrug was projected to be close to 2 years at pH 7.4 and 25 °C. In addition, the prodrugs of cinnarizine and loxapine have been shown to be substrates for alkaline phosphatase, an enzyme found throughout the human body, and revert to the parent compound in its presence. The results from these evaluations demonstrate that the derivatives examined have many of the ideal properties required for potential clinical application.

Introduction

In separate reports, the synthesis and biological evaluation of a novel prodrug approach for improving the water solubility of tertiary amine containing drugs was described.[1,2] A representation of the prodrug strategy is shown in Scheme 1. The parent tertiary amine containing drug **1** is chemically modified to produce the quaternary, water-soluble, prodrug **2**. The prodrug releases the parent tertiary amine in vivo through a two-step bioreversion process. The first step (k_1, rate determining step) in this bioreversion involves a dephosphorylation (phosphatase-catalyzed) to give the resultant hydroxymethyl quaternary ammonium intermediate **4** and inorganic phosphate (**3**). This intermediate **4** is highly unstable at neutral pH and spontaneously breaks down (k_2) to give the parent tertiary amine **1** plus formaldehyde (**5**).

Drugs are most often identified by their ability to interact with target receptors, and their physicochemical properties are initially of secondary concern. With a drug exhibiting poor aqueous solubility, the safe and efficient delivery to the site of action may be an obstacle.[3,4] The solubility behavior of weakly basic drugs such as tertiary amine containing drugs is dependent upon individual molecular properties and the environment in which the drug finds itself. Generally, an ionized molecule is more water-soluble than an un-ionized form, and aqueous formulations are sometimes designed at low pH to utilize the greater solubility of the ionized species.[5] With weakly basic amines (low pK_a), the pH necessary for acceptable solubility may be too acidic to be of practical use. In addition, the intrinsic solubility of the amine salt may be low. This being the case, other means for improving the solubility are often used, such as the addition of cosolvents[6] or complexing agents.[7] Whether using pH extremes and/or organic solvents to improve the solubility, the risk of drug precipitation at the injection site is high for parenterally administered drug.[8,9] This is due to pH changes and/or dilution of cosolvent.



Scheme 1—Illustration of the Novel Prodrug Strategy.

Producing quaternary ammonium prodrugs (of tertiary amine containing drugs) to improve the solubility has been attempted.[10−13] The toxic nature of many quaternary amines[14−16] along with the superior chemical stability of derivatives resulting from simple *N*-alkylation may be responsible for the lack of favorable results.

The shelf life of fosphenytoin (**9-P**), a water soluble prodrug of phenytoin, has been shown to be dictated by the time it takes for phenytoin to precipitate from the aqueous formulation at neutral pH.[17] Likewise, the stability-limiting factor for a formulation containing *N*-phosphonooxymethyl prodrugs would be expected to be the same and not simply be the time for some percentage (i.e., 10%) loss of prodrug.

This report also serves to confirm the in vitro enzymatic lability of selected prodrugs in the presence of alkaline

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10.1021/js9803813 CCC: $18.00
Published on Web 07/09/1999

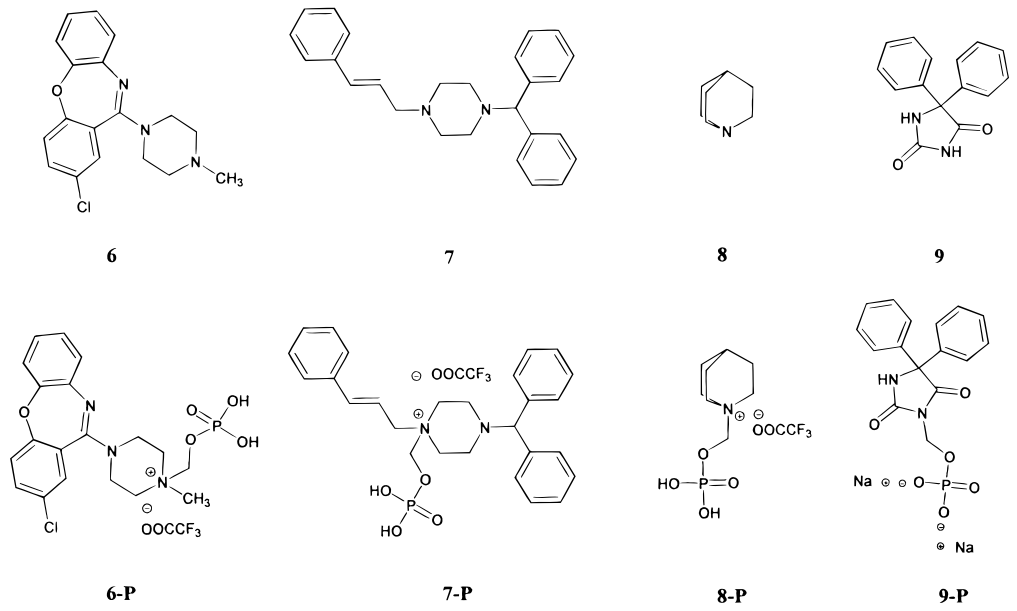


Figure 1—Structures of loxapine (**6**), *N*-phosphonooxymethyl loxapinium trifluoroacetate (**6-P**), cinnarizine (**7**), *N*-phosphonooxymethyl cinnarizinium trifluoroacetate (**7-P**), quinuclidine (**8**), *N*-phosphonooxymethyl quinuclidinium trifluoroacetate (**8-P**), phenytoin (**9**), and fosphenytoin (**9-P**).

phosphatase. Alkaline phosphatase is found throughout the body and is mainly associated with membranes of the intestine, placenta, bone, liver, and kidney in high concentration.[18,19] As mentioned, these prodrugs are designed to be substrates for phosphatases in vivo, which would trigger a rapid chemical breakdown releasing the parent tertiary amine.

Structures of the compounds examined in this work are depicted in Figure 1.

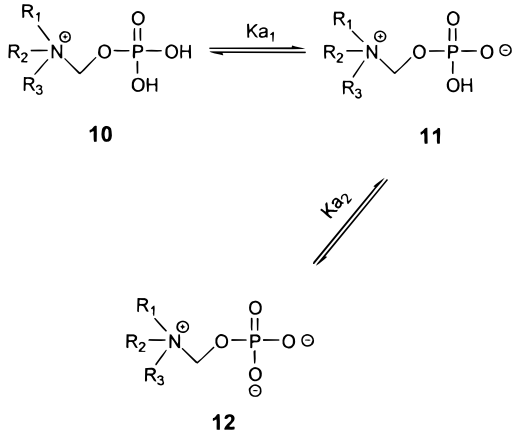
Experimental Section

Materials—**6-P**, **7-P**, and **8-P** were synthesized from a previously described synthetic procedure.[1] Loxapine succinate was obtained from Research Biochemicals Incorporated (Natick, MA). **9-P** was obtained from Parke-Davis Pharmaceuticals (Ann Arbor, MI). Human placental alkaline phosphatase, Type XVII (4.4 units/mg) was obtained from Sigma Chemical Company (St. Louis, MO). All other chemicals were obtained from conventional sources and were used without further purification.

pK_a Determination (Potentiometric)—A 0.01 M quinuclidine prodrug aqueous solution (dihydrogen phosphate form) was titrated using a buret containing a 0.1 N sodium hydroxide volumetric standard solution. The pH was recorded with a calibrated Corning pH/ion analyzer (Corning Incorporated, Corning, NY) after each 0.25 mL addition of NaOH. This experiment was conducted at 25 °C, and all solutions were purged with dry nitrogen gas. The pK_a was calculated from the titration data according to the method of Albert and Seargent.[20]

pK_a Determination (^{31}P NMR)—A 0.25 mmol quantity of the prodrug was dissolved in a 10% v/v D_2O in H_2O solution to prepare a stock solution of 10 mL total volume. Samples spanning the expected pK_a were made by adding minute volumes of a 0.1 N NaOH aqueous solution and recording the pH. Aliquots (0.5 mL) were withdrawn from the stock solution after each pH measurement, transferred to NMR tubes, capped, and frozen at −20 °C until analysis at 25 °C. Spectra were recorded from a Bruker AM 500 MHz NMR spectrophotometer that was tuned to the ^{31}P nucleus. The change in chemical shift was recorded as a function of pH. An insert tube, containing 30% H_3PO_4, was inserted into each NMR tube prior to analysis to serve as an internal reference for the ^{31}P chemical shift of the prodrugs.

The equilibrium for the second ionization of the prodrugs' phosphate monoester (K_{a2}) is represented in Scheme 2. Ignoring the first ionization (K_{a1}), the fraction of prodrug in the zwitterionic form (f_Z) and the fraction in the net anionic form (f_A) are expressed



Scheme 2—Possible Ionic Species of the *N*-phosphonooxymethyl Prodrugs.

in eqs 1 and 2, respectively, where $[H^+]$ represents hydronium ion

$$f_Z = \frac{[H^+]}{[H^+] + K_{a2}} \quad (1)$$

$$f_A = \frac{K_{a_2}}{[H^+] + K_{a2}} \quad (2)$$

molar concentration. The observed chemical shift (δ_{obs}) of the ^{31}P signal is expressed in eq 3, where δ_Z and δ_A represent the chemical

$$\delta_{obs} = f_Z\delta_Z + f_A\delta_A \quad (3)$$

shift for the zwitterionic and net anionic prodrug, respectively. Substituting eqs 1 and 2 into eq 3 gives eq 4. The experimental

$$\delta_{obs} = \frac{[H^+]\delta_Z + K_a\delta_A}{[H^+] + K_a} \quad (4)$$

data was fit to eq 4 using SigmaPlot graphics software (SPSS Inc., Chicago, IL) to estimate values for K_{a2}.

Solubility Determinations—Loxapine succinate was converted to the free base before conducting experiments. All experimental solutions contained 0.05 M buffer and were adjusted to

an ionic strength of 0.2 with NaCl. The pH values with each buffer composition are listed: pH 3.24, HCl/CH_3COONa; pH 3.96 and 4.96, CH_3COOH/CH_3COONa; pH 5.82−7.94, NaH_2PO_4/Na_2HPO_4; pH 8.95 and 9.98, H_3BO_3/NaOH. To glass containers were added 0.2 mL of buffer for the **6-P** samples and 2 mL for the samples of **6**. Each buffered solution was saturated with **6** or **6-P**. The vials were then capped, sonicated, and vortexed prior to submersion in a constant temperature shaking water bath at 25 °C. The samples were shaken at a rate of 100 shakes/min for 24 h. The samples were then centrifuged, and the clear supernatants were diluted and analyzed by HPLC for **6**/**6-P**.

Chemical Stability Evaluations—All experimental solutions contained 0.05 M buffer and were adjusted to an ionic strength of 0.2 with NaCl. The pH values along with their buffer composition are listed: pH 2.05, HCl; pH 3.27, HCl/CH_3COONa; pH 3.94 and 4.95, CH_3COOH/CH_3COONa; pH 5.82−7.94, 8.36, NaH_2PO_4/Na_2HPO_4. Stock solutions for each pH were prepared by dissolving approximately 0.2 mg of **6-P** in 10 mL of buffer. The solution was then assayed for initial prodrug concentration. One-milliliter aliquots were taken and separated into nine glass ampules and sealed. The samples were then submerged in a constant temperature water bath at 65, 75, or 85 °C until analysis. At predetermined times the ampules was opened and mixed with 0.5 mL of acetonitrile to dissolve any precipitate. Each solution was then assayed for prodrug content by HPLC. Individual estimations of k_{obs} were obtained in triplicate by curve-fitting the experimental data to eq 5 (SigmaPlot, SPSS Inc., Chicago, IL), where A is the

$$A = A_0\, e^{-k_{obs}t} \tag{5}$$

prodrug concentration, A_0 is the initial prodrug concentration, k_{obs} is the first-order rate constant for loss of prodrug, and t is time. The loss of prodrug was followed for at least one half-life for all determinations.

In Vitro Enzymatic Lability Evaluations—All experiments involving alkaline phosphatase were performed in a pH 10.4 glycine buffer at 37 °C. The buffer solution contained 1 mM $ZnCl_2$, 1 mM $MgCl_2$, and 0.1 M glycine. The final pH of the buffer was adjusted to pH 10.4 with additions of 2 N NaOH.

For the Michaelis−Menten kinetic evaluation, an alkaline phosphatase stock solution was prepared at a concentration of 149.1 μg/mL (656.0 units/L) in glycine buffer. Four stock solutions of **6-P** (815.3, 163.0, 81.5, and 16.3 μM) were prepared in glycine buffer. To 200 μL of each **6-P** stock solution in a shaking water bath maintained at 37 °C was added 120 μL of the alkaline phosphatase stock solution (at 37 °C). The final concentration of alkaline phosphatase was 245.9 units/L, and the final concentrations of **6-P** were 509.5, 101.9, 51.0, and 10.2 μM. Each reaction was quenched and diluted with addition of 192 μL of a 0.2 N acetic acid solution, 250 μL of acetonitrile, and 232 μL of HPLC mobile phase at predetermined time points (t = 0, 5, 15, and 20 min). Each solution was then vortexed for 30 s, injected into the HPLC, and assayed for **6**.

The initial linear appearance of loxapine was plotted as a function of time for each initial loxapine prodrug concentration following alkaline phosphatase addition. The slope of each was recorded as the initial rate of the reaction. The calculation of Michaelis−Menten parameters was done through plotting the data according to the Lineweaver−Burke equation (eq 6), where V is

$$\frac{1}{V} = \frac{K_m}{V_{max}}\frac{1}{S} + \frac{1}{V_{max}} \tag{6}$$

the initial rate of the reaction, S is the initial concentration of the prodrug, K_m is the Michaelis−Menten constant, and V_{max} is the maximum velocity of the reaction. The slope of this linear plot is equivalent to K_m/V_{max}, and the y-intercept is equal to $1/V_{max}$. From these two relationships the K_m and V_{max} were calculated.

For the enzymatic lability comparisons, stock solutions of alkaline phosphatase (588.7 units/ml), **7-P** (815.9 μM), **6-P** (815.3 μM), and **9-P** (803.6 μM) were prepared in glycine buffer. To 200 μL of prodrug stock solution in a shaking water bath maintained at 37 °C was added 120 μL of the alkaline phosphatase stock solution (at 37 °C). The final concentration of alkaline phosphatase was 220.1 units/L and the final **7-P**, **6-P** and **9-P** concentrations were 509.9, 509.5, and 502.3 μM, respectively. The reaction was quenched and diluted with 100 μL of a 0.4 N acetic acid solution and 200 μL of acetonitrile at predetermined time points (t = 0, 10, 20, 30, 40, and 60 min.). After quenching, each solution was vortexed for 30 s, injected into the HPLC, and assayed for prodrug.

HPLC Conditions—The HPLC system hardware used for all compounds consisted of a Shimadzu LC-6A pump (Kyoto, Japan), a Shimadzu RF-535 fluorescence detector, a Shimadzu SPD-6A UV spectrophotometer, a Shimadzu CR-601 integrator, and Rheodyne 7125 injector (Cotati, CA).

For the analysis of **6**/**6-P**, the mobile phase consisted of acetonitrile (32% v/v) and a 25 mM ammonium phosphate monobasic buffer solution adjusted to pH 3.8 with phosphoric acid (68% v/v). This was pumped at a flow rate of 0.9 mL/min. The injection volume was 20 μL, and the detection was performed by absorbance at 254 nm. The column used for analysis was a Waters Symmetry C-18 150 mm × 4.6 mm with 5 μm particle size (Milford, MA). The retention times were 3.5 and 4.5 min for **6-P** and **6**, respectively.

For the analysis of **7-P**, the mobile phase consisted of acetonitrile (40% v/v) and a 25 mM potassium phosphate monobasic buffer solution adjusted to pH 3.8 with phosphoric acid (60% v/v). The entire mobile phase contained 0.5 mM tetrabutylammonium dihydrogen phosphate (0.5 mM). This was pumped at a flow rate of 1 mL/min. The injection volume was 20 μL, and the detection was by absorbance at 254 nm. The column used for analysis was a Chemco Nucleosil C-18 150 mm × 4.6 mm with 5 μm particle size (Osaka, Japan). The retention time of **7-P** was 4 min.

For the analysis of **9-P**, the mobile phase consisted of acetonitrile (35% v/v) and a 25 mM potassium phosphate monobasic buffer solution adjusted to pH 3.8 with phosphoric acid (65% v/v). This was pumped at a flow rate of 1.1 mL/min. The injection volume was 50 μL, and detection was by fluorescence using an excitation wavelength of 260 nm and an emission wavelength of 315 nm. The column used for analysis was a C18 ODS Hypersil 15 cm × 4.6 mm with 5 μM particle size. The retention time of **9-P** was 4 min.

Results and Discussion

Solubility Evaluation—For the *N*-phosphonooxymethyl prodrugs to be useful for parenteral delivery, they must have sufficient water solubility at the desired formulation pH, preferably in the physiologically acceptable pH range. Limited quantities of prodrug materials prevented us from fully elucidating the pH solubility behavior of the prodrugs. Some limited estimates and predictions were made, however.

The prodrugs have a quaternary center that bears a cationic charge at all pH values, and the phosphate monoester has an ionization scheme depicted in Scheme 2. The phosphate ester exists in equilibrium between the diacidic **10**, monobasic **11**, and the dibasic species **12**, and the fraction of each species present in solution is a function of the pK_a values and the pH of the solution. The overall net charge of the prodrug will be zero when the phosphate ester is predominantly monobasic **11**. Accordingly, at this pH, the water-solubility of this prodrug species would be expected to be the least. Lowering or raising the pH several units from this pH should increase the solubility of the prodrug, as the net charge of the prodrug will become plus one and minus one, respectively.

The pK_{a_2} of the phosphate ester is important considering its potential influence on the solubility, chemical stability, and effectiveness as an enzymatic substrate in the physiological pH range. The pK_{a1} is expected to be less than one and of little physiological importance, except that it is quite acidic. The pK_{a2} was determined by ^{31}P NMR and was found to be 4.7 and 4.9 for **6-P** and **8-P**, respectively. A plot of the ^{31}P NMR chemical shift as a function of pH for **8-P** is shown in Figure 2. The pK_{a2} for **8-P** was 5.0 by potentiometric titration, which was in close agreement with the value determined by ^{31}P NMR.

The measured solubility of **6-P** at pH 3 was 290.5 ± 2.9 mg/mL, which is expected to be a reasonable estimate of

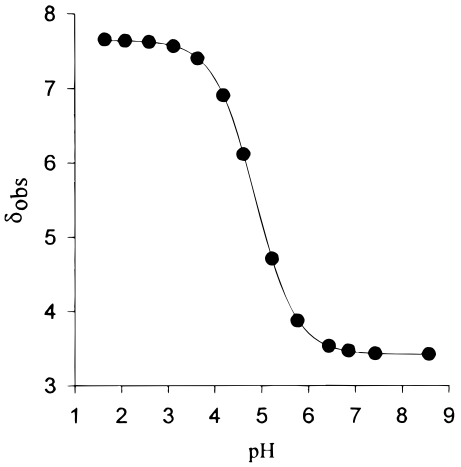


Figure 2—Plot of the observed chemical shift of the ^{31}P NMR signal from **8-P** as a function of pH. The solid line is the curve fit to the experimental data.

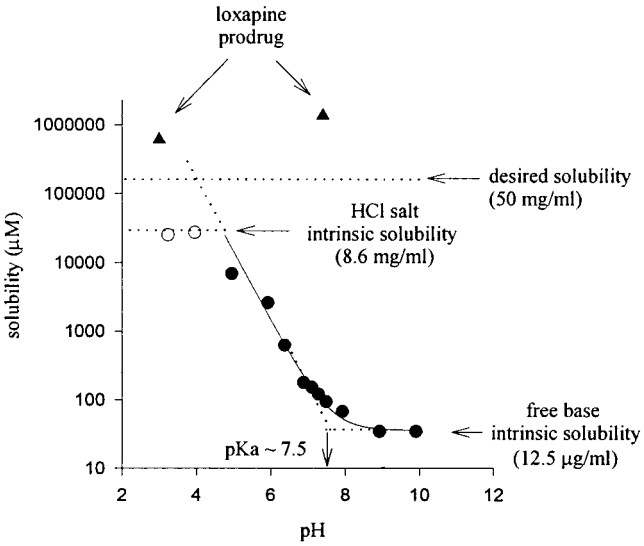


Figure 3—Plot of the water solubility of **6** (●, ○) and **6-P** (▲) as a function of pH.

the solubility of **6-P** in the least soluble zwitterionic form. When the pH was raised to 7.4, the solubility of the prodrug more than doubled (648.2 ± 0.6 mg/mL). Figure 3 compares the solubility behavior of **6-P** relative to **6** as a function of pH. The solid points represent the experimentally determined solubilities for **6** as a function of pH. The line through these points is a theoretical plot of the solubility profile obtained through curve fitting the experimental data to eq 7, where S_t is the total measured solubility of **6**,

$$\log S_t = \log\left(\frac{[H^+]}{K_a} + 1\right) + \log S_o \qquad (7)$$

$[H^+]$ is the hydrogen ion molar concentration, K_a is the acid dissociation constant for the protonated amine, and S_o is the intrinsic solubility of the free base. Estimates of S_o and pK_a were obtained through curve fitting and were found to be 12.6 μg/mL and 7.5, respectively.

Lowering the pH is a common method used for improving the solubility of weakly basic drugs through ionization. Even at pH 3.2, however, the solubility of **6** only reaches 8.2 mg/mL, which is still distant from the desired formulation concentration of 50 mg/mL. **6-P** offers over a 15 000-fold increase in solubility compared to the free base at pH 7.4. This translates to prodrug solubilities that are over

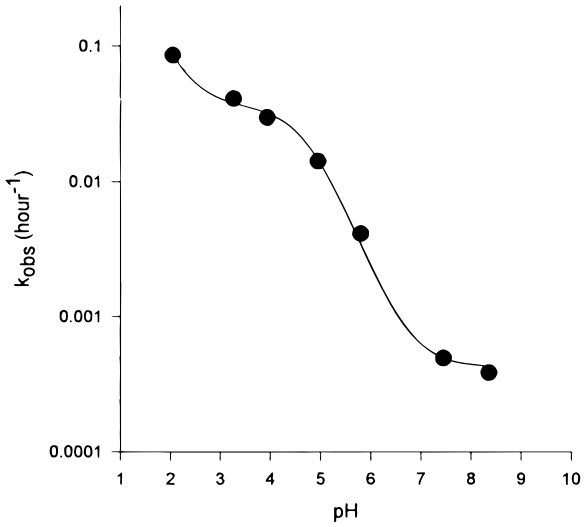


Figure 4—Plot of the observed rate constant (k_{obs}) for loss of **6-P** as a function of pH at 65 °C. The solid line is the curve fit to the experimental data.

nine times higher than those used in the im formulation without the need for cosolvents.

Chemical Stability Evaluation—The prodrugs must have adequate chemical stability in any formulation, especially in ready-to-use parenteral dosage forms. The pH rate profile for **6-P** at 65 °C is shown in Figure 4. The pH of maximum stability occurs in the neutral pH range, which is ideal for formulation from a physiological viewpoint.

The prodrug can exist in three distinct ionic species (see Scheme 2). Because each of these is potentially reactive, several mechanistic pathways for the degradation are possible. A theoretical profile, in reasonable agreement with the observed profile, was constructed according to eq 8, where k_{obs} is the observed rate constant for loss of

$$k_{obs} = \frac{k_o[H^+]^2 + k'_oK_{a1}[H^+] + k''_oK_{a1}K_{a2}}{[H^+]^2 + [H^+]K_{a1} + K_{a1}K_{a2}} \qquad (8)$$

prodrug while K_{a1} and K_{a2} are the first and second acid dissociation constants for the phosphate ester. The k_o, k'_o, and k''_o variables represent the rate constants for water-catalyzed or spontaneous hydrolysis of the diacidic **10**, monobasic **11**, and dibasic **12** phosphate species of the prodrug, respectively. Values for k_o, k'_o, k''_o, and K_{a2} were estimated through curve fitting while the value for K_{a1} was fixed at 0.4 (estimated value). The parameters used to generate the theoretical profile in Figure 4 were as follows: $k_o = 2.32 \pm 0.21$ h^{-1}; $k'_o = (3.62 \pm 0.24) \times 10^{-2}$ h^{-1}; $k''_o = (4.24 \pm 1.95) \times 10^{-4}$ h^{-1}; $K_{a1} = 0.4$; $K_{a2} = (1.73 \pm 0.36) \times 10^{-5}$. The kinetically determined pK_{a2} value of 4.76 ± 0.08 at 65 °C was in good agreement with the value of 4.7 ± 0.01 determined by ^{31}P NMR at 25 °C, especially considering the temperature differences.

The shape of this profile is consistent with other phosphate monoesters for which the increased rate of hydrolysis at low pH is consistent with the higher reactivity of the monobasic phosphate species.[21−26]

A concentrated parenteral formulation of a water-soluble prodrug (of a sparingly soluble drug) might produce sparingly soluble products following degradation. The stability-limiting factor, therefore, may be parent drug precipitation. The precipitation is expected to occur far in advance to the time for 10% loss of prodrug ($t_{90\%}$) often used as an indicator of shelf life of pharmaceuticals. Figure 5 shows Arrhenius plots for the loss of **6-P** at pH values 5.8, 7.45, and 8.36 at temperatures of 65, 75, and 85 °C. From the slope of these plots, Arrhenius activation energies were calculated to be

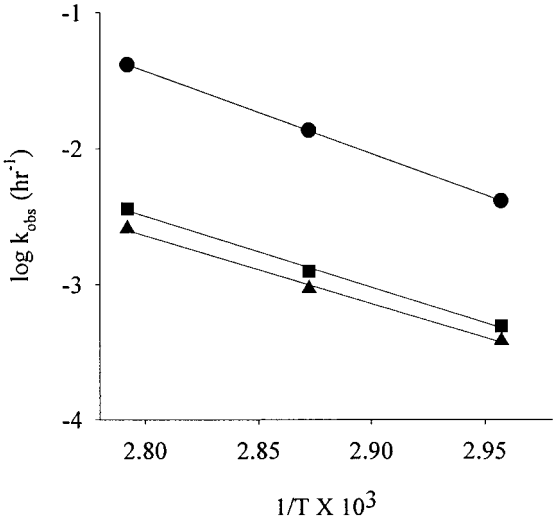


Figure 5—Arrhenius plots for **6-P** degradation at pH values 5.8 (●), 7.4 (▲), and 8.36 (■). The temperatures studied were 65, 75, and 85 °C. Error bars are hidden by the symbols.

Table 1—Arrhenius Plot Extrapolations of the Mean (±SD) k_{obs}, Half-Life, and $t_{90\%}$ for *N*-Phosphonooxymethyl Loxapine Degradation at 25 °C at Several pH Values

pH	k_{obs} (hr^{-1} 10^6)	$t_{1/2}$ (years)	$t_{90\%}$ (years)
5.36	16.2 ± 0.1	4.8 ± 0.1	0.7 ± 0.1
7.45	3.7 ± 0.7	21.1 ± 5.3	3.2 ± 0.8
8.36	3.6 ± 0.7	21.7 ± 5.6	3.3 ± 0.8

27.7 ± 0.2, 23.9 ± 0.8, and 22.9 ± 0.7 kcal/mol at pH values 5.8, 7.4, and 8.3, respectively. These values were then used to predict the $t_{90\%}$, half-life ($t_{1/2}$), and k_{obs} for **6-P** degradation at 25 °C listed in Table 1.

To estimate the shelf life based upon precipitation of **6**, the following example is used. An im formulation of **6-P** at a concentration of 72 mg/mL (equivalent to 50 mg/mL of **6**) at pH 7.4 could only tolerate 0.06% degradation before the solubility of **6** (30 μg/mL) is exceeded. Using the k_{obs} from Table 1 at pH 7.4 to calculate the time for 0.06% degradation ($t_{99.94\%}$) gives a value of 6.7 days, which is drastically shorter than the 3.2 year $t_{90\%}$ estimate from Table 1. This calculation assumes that upon degradation **6-P** is quantitatively converted to **6** and that the solution of **6-P** does not alter the solubility of **6**. Fortunately, a solution of **6-P** at a concentration of 72 mg/mL does in fact improve the solubility of **6**. The intrinsic solubility of **6** at pH 11.8 in a solution containing 72 mg/mL of **6-P** ($n = 1$, as a result of lack of prodrug material) was found to be 2.7 mg/mL. Taking this solubility change into account, a possible shelf life ($t_{94.6\%}$) for **6-P** at pH 7.4 would be about 1.7 years. The solubility of **6** would be expected to be higher than 2.7 mg/mL at pH 7.4 compared to pH 11.8. Therefore, the shelf life of 1.7 years is probably an underestimate.

In Vitro Evaluation—For the derivatives to behave as prodrugs, they must undergo a rapid and quantitative enzymatic bioreversion. The prodrugs are designed to undergo a two-step bioreversion process to give the parent amine, formaldehyde, and inorganic phosphate (Scheme 1). The first step in bioreversion is a rate-determining, enzyme-catalyzed dephosphorylation step ($\mathbf{k_1}$) followed by a fast chemical breakdown ($\mathbf{k_2}$) of the hydroxymethyl derivative (**4**).

In an earlier report, **6-P** and **7-P** were shown to be substrates for alkaline phosphatase. Further evaluation of this enzymatic process can provide information on the kinetics of hydrolysis. The hydrolysis kinetics of **6-P** were

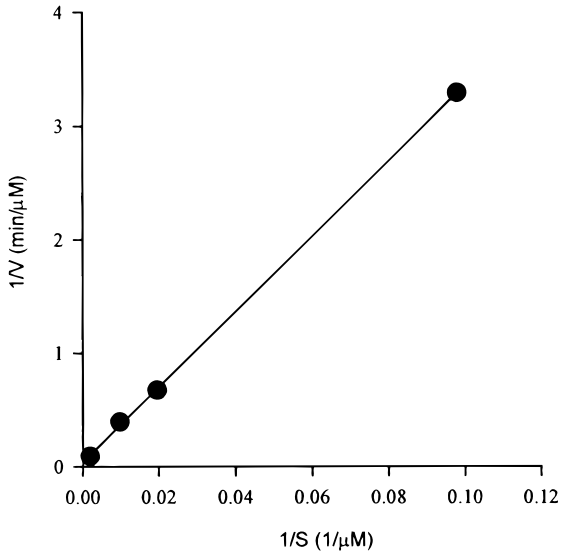


Figure 6—Lineweaver–Burke plot for the enzymatically catalyzed dephosphorylation of **6-P** with alkaline phosphatase at pH 10.4 and 37 °C.

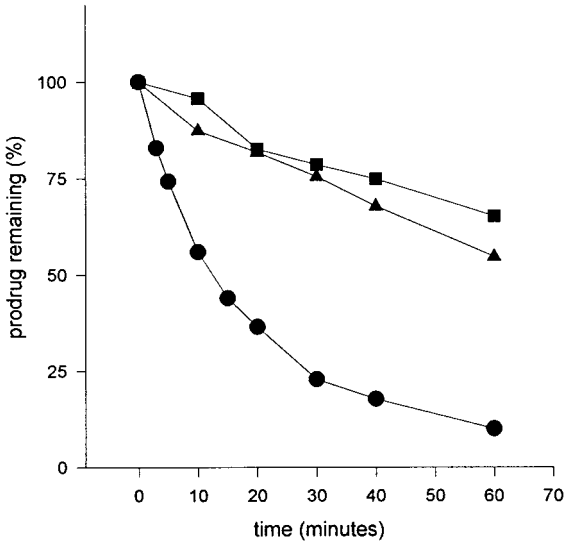


Figure 7—Plot of percent prodrug remaining as a function of time for **9-P** (●), **6-P** (▲), and **7-P** (■) in the presence of alkaline phosphatase.

found to be consistent with a Michaelis–Menten kinetic model similar to other phosphate monoesters reported in the literature.[27,28]

The values for K_m and V_{max} were 872 μM and 26 μM/min obtained from a Lineweaver–Burke plot shown in Figure 6. The value for k_{cat} (0.9 s^{-1}) was obtained by dividing V_{max} by the molar enzyme concentration. The value for k_{cat}/K_m (the second-order rate constant for catalysis) was 1063 M^{-1} s^{-1}. This value is similar to[27] and significantly lower[29] than values others have reported, but comparing these k_{cat}/K_m values may be somewhat meaningless because the enzyme source and experimental conditions of temperature and pH were different.

Fosphenytoin (Cerebyx, **9-P**) is a commercially available phosphate prodrug of phenytoin that requires an in vivo enzyme-catalyzed dephosphorylation step to release phenytoin.[30] Fosphenytoin is rapidly converted to phenytoin in humans with a half-life of approximately 8 min.[31] A comparison of the in vitro labilities of the prodrugs in the presence of isolated alkaline phosphatase would provide some insight as to how the cinnarizine and loxapine prodrugs might behave in vivo. Figure 7 is a plot of % prodrug remaining versus time for **6-P** and **7-P** compared

to fosphenytoin with isolated alkaline phosphatase. Each of the prodrugs was found to be chemically stable under the experimental conditions when the solution was devoid of enzyme. From this plot it appears that enzyme-catalyzed breakdown of **7-P** and **6-P** are similar, and **9-P** appears to be catalyzed at a significantly faster rate. The rapid in vivo conversion of the *N*-phosphonooxymethyl prodrugs in rats and dogs would not have predicted this trend; however, the inability of in vitro trends to predict in vivo results is not uncommon. It also cannot be ruled out that alkaline phosphatase may not exclusively be responsible for the catalytic dephosphorylation; catalytic promiscuity displayed by other enzymes (i.e., esterases) may be responsible for a significant fraction of the in vivo enzymatic reversion.

Conclusion

The *N*-phosphonooxymethyl prodrug approach has been described as a novel method for improving the water solubility of tertiary amine containing drugs. Separate reports have demonstrated the synthetic feasibility and rapid and quantitative prodrug to parent drug conversion in rats and dogs, which are essential characteristics for a clinically useful prodrug. This work has confirmed the prodrugs to have dramatically improved water solubility behavior and very good chemical stability at physiological pH values. The prodrugs have also been shown to be substrates for alkaline phosphatase, which triggered the release of the parent molecule. These results suggest that this approach for improving the water solubility of tertiary amine containing drugs is very promising and deserves further evaluation.

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Acknowledgments

The authors thank the American Foundation for Pharmaceutical Education Fellowship and a National Cancer Institute Training Grant for financial support. Additional support was given by the Center for Drug Delivery Research and by Kansas Technology Enterprise Corporation through the Centers of Excellence program.

JS9803813