# A Novel Prodrug Approach for Tertiary Amines. 3. In Vivo Evaluation of Two *N*-Phosphonooxymethyl Prodrugs in Rats and Dogs

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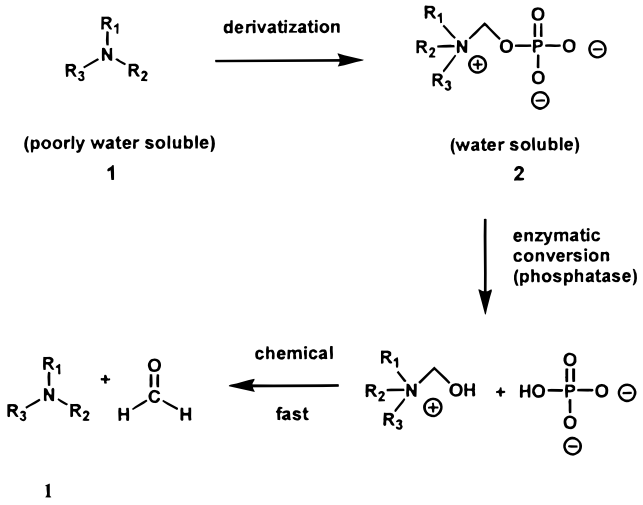
Received September 21, 1998. Final revised manuscript received May 21, 1999.
Accepted for publication May 26, 1999.

**Abstract** □ *N*-Phosphonooxymethyl derivatives of tertiary amine containing drugs have been identified as a novel prodrug approach for improving aqueous solubility. The in vivo reversion of two prodrugs to the corresponding parent compounds following iv and im administration to rats and dogs was investigated. Equimolar doses of parent drugs (loxapine or cinnarizine) and the corresponding prodrugs were each administered via a rapid iv infusion to rats and dogs. Equimolar doses of loxapine and its prodrug were each administered im to rats only. Blood samples were collected over 12 h, and plasma was assayed for both parent drug and intact prodrug by HPLC. Comparison of the plasma AUC for the parent drugs following administration of the parent drugs and prodrugs allowed estimation of the apparent bioavailability of parent drug from prodrug dosing. Plasma levels of the prodrugs fell below the limit of detection 5 min after iv infusion with an approximate half-life of 1 min. The mean AUCs following iv and im dosing of parent drugs were not statistically different from the parent drug AUCs obtained after prodrug dosing. The results are consistent with rapid and quantitative prodrug to parent drug reversion following administration of the phosphonooxymethyl prodrugs to the rats and dogs. This information, together with previous studies on the synthesis and physicochemical evaluation of the prodrugs, suggests that this novel prodrug strategy is a very promising approach for overcoming solubility limitations seen with many tertiary amine containing drugs at physiological pH values.

## Introduction

The synthesis and the physicochemical and in vitro enzymatic evaluation of a novel prodrug approach for improving the water solubility of tertiary amine containing drugs has been described.[1,2] A schematic of this prodrug strategy is shown in Scheme 1. The tertiary amine drug **1** is chemically derivatized to produce the quaternary water-soluble derivative **2**. This derivative was designed to behave as a prodrug and release parent tertiary amine following in vivo administration. This bioreversion process is thought to occur through two steps, the first step being a rate-determining (enzyme-catalyzed) dephosphorylation.

The *N*-phosphonooxymethyl prodrugs of two drugs, cinnarizine[3−7] and loxapine,[8] were selected for the in vivo evaluation of this prodrug strategy. Both drugs are sparingly water soluble. Cinnarizine is not formulated as an injectable, whereas loxapine (formulated for im use) requires a potentially toxic cocktail of propylene glycol and polysorbate 80.[9] Each of the prodrugs can be formulated in pure water at physiological pH, which might eliminate the vehicle-related toxicities encountered with poorly soluble drugs.




**Scheme 1**—Illustration of the *N*-Phosphonooxymethyl Prodrug Strategy.

The objective of the work described in this paper was to show that the *N*-phosphonooxymethyl derivatives of loxapine and cinnarizine quickly and quantitatively revert to parent compounds after administration to rats and dogs. To realize quantitative parent drug delivery, the rate of prodrug reversion must be optimal. Assuming that the prodrug does not extensively distribute into tissues, the prodrug can be removed by elimination ($k_e$) and conversion ($k_r$) to parent drug. If $k_e$ is fast or even comparable to $k_r$, then quantitative recovery of parent drug would not occur. If $k_r$ was large relative to $k_e$, quantitative delivery of parent drug would be realized and the pharmacokinetic profiles would be identical to those resulting from direct delivery of parent compound. Theoretically, a conversion that is too rapid may lead to some complications; however, in most cases a fast $k_r$ is desirable both for quantitatively delivering parent drug and for reducing the risk of a pharmacological effect by the prodrug, if the prodrug is not completely inert.

## Materials and Methods

**Chemicals**—Cinnarizine was obtained from Sigma Chemical Co. (St. Louis, MO). Loxapine succinate was obtained from Research Biochemicals Incorporated (Natick, MA). *N*-Phosphonooxymethyl derivatives of cinnarizine (**7-P**) and loxapine (**6-P**) (Figure 1) were synthesized as previously described.[1] $(SBE)_{7M}$-$\beta$-cyclodextrin (Captisol) was obtained from Cydex, Inc. (Overland Park, KS). Methyl *tert*-butyl ether was obtained from EM Science (Gibbstown, NJ). Isoflurane (Forthane) was obtained from Abbott

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10.1021/js980382v CCC: $18.00
*Published on Web 07/09/1999*

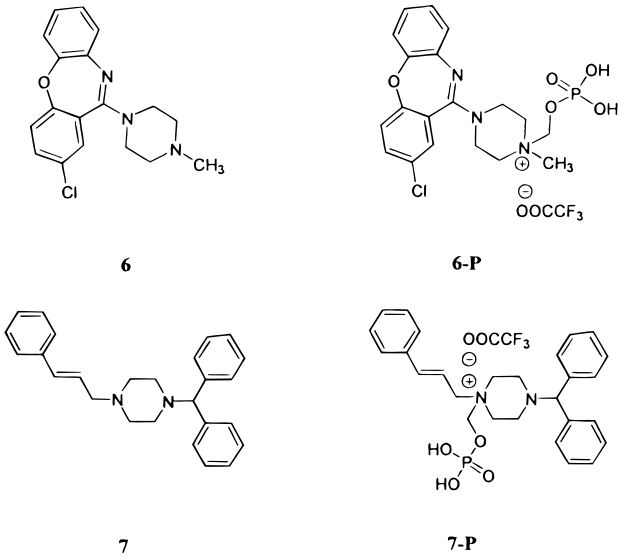


**Figure 1**—Structures of loxapine (**6**), *N*-phosphonooxymethyl loxapinium trifluoroacetate (**6-P**), cinnarizine (**7**), and *N*-phosphonooxymethyl cinnarizinium trifluoroacetate (**7-P**).

Australasia (Kurnell, Australia). All other chemicals were obtained from conventional sources.

**Experimental Procedure, Rat Study**—Male Sprague-Dawley rats (weight range 250−300 g) were fasted for 24 h prior to experimentation. The rats were anesthetized with a continuous 2% dose of isoflurane given by inhalation with a 95% $O_2$, 5% $CO_2$ carrier gas flowing at 1 L/minute. The rats were implanted with in-dwelling polyethylene cannulae (i.d. 0.58 mm, o.d. 0.965 mm) inserted into the jugular vein and the carotid artery for drug administration and blood sampling, respectively. The cannulae were sutured into position and then exteriorized through a subcutaneous tunnel made from the frontal neck incision to the back of the neck. The rats were then connected to a harness that secured the cannula while allowing the rat free mobility and access to water. The rats awoke 10 min after the cessation of anesthesia and were allowed to recover from the surgical procedure for at least 12 h before dosing. Intravenous doses were administered over 3 min into the jugular cannula, after which the cannula was flushed with 1 mL of normal saline containing 20 units/mL of heparin sodium. Intramuscular doses were administered into the hind leg using a 25 gauge needle. Blood samples (200 $\mu$L) for parent drug and prodrug analysis were taken from the carotid cannula before dosing and at predetermined time points following drug/prodrug administration. The sampling times for **6**/**6-P** were 0, 5, 15, and 30 min and 1, 2, 3, and 4 h postinfusion and 1, 5, 15, 30, and 45 min and 1, 2, 4, 6, and 8 h after the im injection. The sampling times for **7**/**7-P** were 0, 5, 10, 20, and 40 min and 1, 2, and 4 h postinfusion. After each blood sample, 200 $\mu$L of heparinized saline (20 units/mL) was infused through the carotid cannula. All blood samples were collected in microcentrifuge tubes containing 100 units of heparin sodium (20 $\mu$L of a 5000 units/mL solution) and were immediately centrifuged at 3000 rpm for 10 min with a Beckman SG-6R series centrifuge (Fullerton, CA). One hundred microliters of plasma was then separated and stored at −20 °C until analysis.

**Experimental Procedure, Dog Study**—The study was conducted as a 3 × 3 randomized crossover study in male beagle dogs (weight range 11.8−17.8 kg). The dogs were fasted for 1 day prior to drug/prodrug administration and were given a 1 week washout period between doses. In-dwelling venous catheters were placed in each front leg, one for dosing and the other for sampling. Blood samples (3 mL) for **6** and **6-P** analysis were taken before dosing and at 0, 5, 15, and 30 min and 1, 2, 4, and 6 h postinfusion. All catheters were flushed with 3 mL of heparinized saline (1 unit/mL) after dosing and after each blood sample. Blood samples were collected into 5 mL collection tubes containing EDTA and were immediately centrifuged at 10 000 rpm for 4 min, and 1.5 mL of plasma was separated and stored at −20 °C until extraction.

**iv and im Formulations**—A solution of **6** for iv infusion was prepared in a cosolvent formulation consisting of 10% (v/v) ethanol, 1.5% (v/v) benzyl alcohol, and 40% (v/v) propylene glycol. This is the commercially used vehicle for iv diazepam[10] and was employed because **6** is not available for iv delivery. A solution of **6** for im delivery was prepared in a cosolvent consisting of 70% (v/v) propylene glycol and 5% (v/v) polysorbate 80 in sterile water for injection, which is the commercially available im injection formulation for loxapine.[11]

A method of formulating **7** for iv infusion was adapted from Järvinen et al.[12] **7** was prepared in a 10 mM sodium phosphate buffer solution containing 37.5 mM of Captisol as a solubilizing excipient. The pH of the solution was lowered to pH 3.5 with addition of 1 M HCl solution. The solution was allowed to stir overnight at room temperature, and the final pH was adjusted to 5.5 with addition of 1 M NaOH.

All prodrugs for all routes of administration were formulated in phosphate buffered saline at pH 6.5 except for **6-P** for im delivery, which was formulated in normal saline. All solutions were passed through a 0.22 $\mu$M nylon filter and assayed for drug/prodrug content just prior to administration.

**Plasma Extractions of 6, 6-P, 7, and 7-P**—One milliliter or 100 $\mu$L plasma samples were obtained from the dog or rat blood, respectively, at specified time points. To the plasma following administration of **6**/**6-P** was added 20 $\mu$L of $H_2O$ and 20% plasma volume of saturated $ZnSO_4$ (in 0.5 N NaOH). To the plasma following administration of **7**/**7-P** was added 20 $\mu$L of acetonitrile−$H_2O$ (50:50) and 20 $\mu$L of 10% (w/v) trichloroacetic acid aqueous solution. To the protein-precipitated plasma was added 2 mL of methyl *tert*-butyl ether. Each sample was subsequently vortexed for one minute and centrifuged at 3000 rpm for 10 min. To assay for **6** or **7**, an aliquot of the organic layer (1.7 mL) was taken and evaporated to dryness with a stream of nitrogen gas. The residue was redissolved in 200 $\mu$L of HPLC mobile phase, vortexed briefly, and injected onto the HPLC. The efficiencies of the extraction procedure were 88% and 83% for **6** and **7**, respectively. To assay for **6-P** or **7-P**, the remaining aqueous phase from the ether extraction was evaporated to dryness under a stream of nitrogen gas (for rat plasma samples) or lyophilized (for dog plasma samples). To the dried residue was added 2 mL of methanol and 40 or 400 $\mu$L of 10% (w/v) trichloroacetic acid aqueous solution for the rat and dog samples, respectively. The mixture was vortexed for 5 min and centrifuged at 3000 rpm for 10 min. An aliquot of the organic layer (1.7 mL) was taken and reduced to dryness under a stream of nitrogen gas. The resulting residue was redissolved in 200 $\mu$L of water and vortexed briefly, after which the solution was filtered through a 0.2 $\mu$M microcentrifuge filter unit and injected into the HPLC. The efficiencies of the extraction procedure were 47% and 70% for **6-P** and **7-P**, respectively.

Stability of all drugs and prodrugs under the previously described conditions was assessed. Drugs **6** and **7** had complete stability for at least 2 h in whole blood. Prodrugs **6-P** and **7-P**, however, had detectable conversion to **6** and **7** in whole blood that was very temperature-dependent. The extent of this conversion was determined by spiking freshly drawn blood with a known concentration of prodrug and assaying for parent drug and prodrug with the described method. The extent of prodrug to drug conversion was less than 10% for both prodrugs within a 2 h period at room temperature, whereas approximately 50% of **6-P** was converted to **6** at 37 °C in the same amount of time. As a result, all blood samples were chilled on ice and processed as quickly as possible to limit ex vivo conversion.

To evaluate intraday and interday precision of the extraction methods, each procedure was repeated five times in 1 day and on 3 separate days with low, medium, and high plasma drug/prodrug concentrations. The percent standard deviations using either peak height or peak area values were generally less than 10% for both intraday and interday evaluations. Linear standard curves ($r^2$ > 0.990) resulted by spiking known amounts of drug/prodrug in blank plasma to achieve plasma concentrations in the range of interest for each compound.

**HPLC Analysis**—The HPLC system for all compounds consisted of a Waters model 510 pump, a Waters 717 autosampler (Milford, MA), a Perkin-Elmer LC240 fluorescence detector (Beaconsfield, Buckinghamshire, England), an LDC Analytical Spectromonitor 3100 variable wavelength detector, a Waters C18 Symmetry column (3.9 × 150 mm, Milford, MA), a Shimadzu CR6A intergrator (Kyoto, Japan), and a column heater (Timberline Instruments Inc., Boulder, CO).

For the analysis of **6**, the mobile phase consisted of acetonitrile (50% v/v) and a 10 mM ammonium dihydrogen phosphate buffer adjusted to pH 7 with ammonium hydroxide (50% v/v). This was pumped at a flow rate of 0.9 mL/min. The injection volume was 150 $\mu$L with detection at 251 nm. Under these conditions, the retention time was 11.4 min.

For the analysis of **6-P**, the mobile phase consisted of acetonitrile (25% v/v) and a 10 mM ammonium dihydrogen phosphate buffer adjusted to pH 3 with phosphoric acid (75% v/v). The solvent was pumped at a flow rate of 0.9 mL/min. The injection volume was 150 $\mu$L with detection at 251 nm. Under these conditions, the retention time was 12 min.

For the analysis of **7**, the mobile phase consisted of acetonitrile (50% v/v) and a 10 mM ammonium dihydrogen phosphate buffer (50% v/v). This was pumped at a flow rate of 0.9 mL/min. The injection volume was 50 $\mu$L with fluorescence detection using an excitation wavelength of 249 nm and an emission wavelength of 311 nm. Under these conditions, the retention time was 7.7 min.

For the analysis of **7-P**, the mobile phase consisted of acetonitrile (30% v/v) and a 10 mM ammonium dihydrogen phosphate buffer (70% v/v). Solvent was pumped at a flow rate of 0.9 mL/min, and the injection volume was 150 $\mu$L. Detection was conducted at 253 nm with an observed retention time of 9 min.

**AUC and Bioavailability Calculations**—After intravenous administration, all concentration versus time profiles were fit to eq 1 using SigmaPlot graphics software (SPSS Inc, Chicago, IL)

$$C = A\,e^{-\alpha t} + B\,e^{-\beta t} \quad (1)$$

with reciprocal concentration weighting. Equation 1 describes the plasma drug concentration as a function of time for a drug that exhibits biexponential pharmacokinetics. The parameters $A$, $\alpha$, $B$, and $\beta$ generated from the curve fit were used to calculate the $AUC_0^\infty$ values.

After intramuscular administration the $AUC_0^t$, where $t$ was the last time point, was estimated using the trapezoidal method,[13] and the area under the last point to infinity was estimated by dividing the last point by the apparent elimination rate constant obtained from the curve fit to the iv data. The area under the tail was combined with $AUC_0^t$ to give estimates of $AUC_0^\infty$.

The apparent bioavailability of the parent drug following administration of the prodrug ($F_{app}$) was calculated by dividing the parent drug AUC following prodrug dosing by that from parent drug dosing as is shown in eq 2. The absolute bioavailability ($F_{abs}$)

$$F_{app} = \frac{\text{Parent Drug AUC (after prodrug dosing)}}{\text{Parent Drug AUC (after parent drug dosing)}} \quad (2)$$

of parent drug following parent and prodrug intramuscular dosing were calculated using eq 3. A one-way ANOVA test was used to

$$F_{abs} = \frac{AUC_0^\infty(\text{im})}{AUC_0^\infty(\text{iv})} \times \frac{\text{Dose(iv)}}{\text{Dose(im)}} \quad (3)$$

compare mean AUCs following drug and prodrug dosing.

## Results and Discussion

In all studies, equimolar doses of drug and prodrug were administered and plasma concentrations of both parent drug and prodrug were recorded as a function of time. Doses between different routes of administration did differ. The area under the concentration versus time profile for parent drug following prodrug administration was compared to that following parent drug administration to quantitatively assess the bioreversion. Table 1 summarizes the mean AUCs from each dosing along with the calculated availability, where relevant. Results are discussed in more detail below.

**Evaluation of 6-P**—Plasma concentration versus time profiles for **6** following equimolar (4.6 $\mu$mol/kg) intravenous infusions of **6** and **6-P** to dogs are shown in Figure 2. Plasma concentrations of **6-P** at 0 and 5 min postinfusion

**Table 1—Summary of AUC Values and Bioavailabilities of 6 and 7 Resulting from Administration of Parent Drugs (6 and 7) and *N*-Phosphonooxymethyl Prodrugs (6-P and 7-P) to Rats and Dogs**

| compd | dose ($\mu$mol/kg) | route | animal ($n$) | AUC[a] (ng·hr/mL ± SE) | $F_{app}$ | $F_{abs}$ |
|---|---|---|---|---|---|---|
| **6** | 4.6 | iv | dog (3) | 663 ± 115 | | |
| **6-P** | 4.6 | iv | dog (3) | 564 ± 75 | 0.86 | |
| **6** | 21.3 | iv | rat (3) | 626 ± 193 | | |
| **6-P** | 21.3 | iv | rat (3) | 711 ± 169 | 1.13 | |
| **6** | 30.5 | im | rat (3) | 661 ± 181 | | 0.70 |
| **6-P** | 30.5 | im | rat (3) | 839 ± 323 | 1.27 | 0.89 |
| **7** | 6.2 | iv | rat (3) | 183 ± 98 | | |
| **7-P** | 6.2 | iv | rat (3) | 166 ± 31 | 0.91 | |
| **7-P** | 18.6 | iv | rat (3) | 333 ± 60 | | |

[a] AUC is for **6** or **7**.

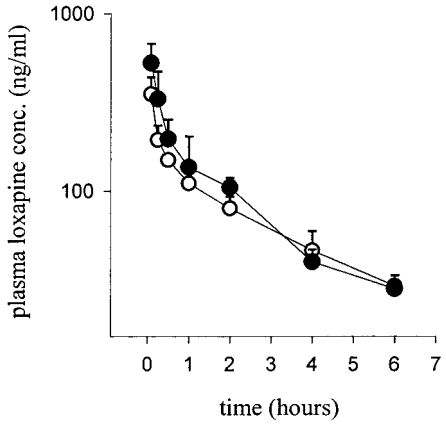


**Figure 2**—Semilog plot of mean (±SD) plasma concentration versus time profiles of **6** following equimolar (4.6 $\mu$mol/kg) 3 min iv infusions of **6** (●) or **6-P** (○) to beagle dogs in a 3 × 3 randomized crossover study.

**Table 2—Summary of Plasma Prodrug Levels following Prodrug Administration to Dogs and Rats[a]**

| compd | dose ($\mu$mol/kg) | route | animal ($n$) | plasma prodrug concn (ng/mL ± SE) 0 min | 5 min |
|---|---|---|---|---|---|
| **6-P** | 4.6 | iv | dog (3) | 11,902 ± 983 | 414 ± 218 |
| **6-P** | 21.3 | iv | rat (3) | 1605 ± 737 | 386 ± 145 |
| **6-P** | 30.5 | im | rat (3) | 0[b] | 0[b] |
| **7-P** | 6.2 | iv | rat (3) | 1388 ± 615 | 96 ± 9 |

[a] After 5 min, prodrug levels were below the limit of detection for all samples collected. [b] Levels were below the limit of detection.

are shown in Table 2. Levels of **6-P** were below the limit of detection (48 ng/mL) following the 5 min sample. The mean AUC values of **6** resulting from dosing of **6-P** and **6** were not significantly different ($\alpha = 0.05$), and the apparent bioavailability of **6** following **6-P** administration was found to be 0.86. Similar results are shown in Figure 3 following equimolar (21.3 $\mu$mol/kg) iv infusions of **6** and **6-P** to rats. Again, the prodrug was rapidly cleared from the plasma, and the mean AUCs of **6** resulting from **6** and **6-P** dosing were not statistically different ($\alpha = 0.05$) and gave a calculated bioavailability of **6** following **6-P** administration of 1.13. Rapid disappearance of the prodrug from the plasma along with the virtual superimposability of plasma parent drug profiles following equimolar iv infusions of parent drug and prodrug are consistent with rapid and quantitative reversion, which was evident for all animals tested.

As **6** is currently formulated for intramuscular injection at a concentration of 50 mg/mL in a predominantly nonaqueous cosolvent (70% v/v propylene glycol and 5% v/v

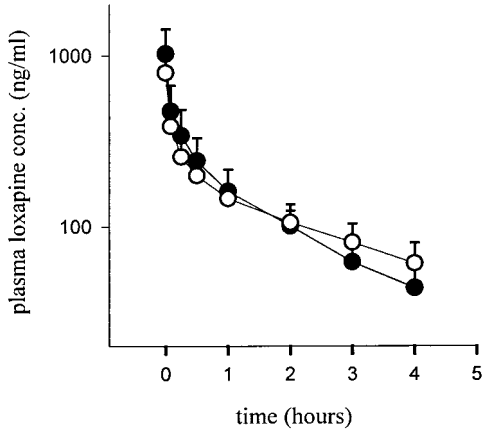


**Figure 3**—Semilog plot of mean (±SD) plasma concentration versus time profiles of **6** following equimolar (21.3 $\mu$mol/kg) 3 min iv infusions of **6** (●) or **6-P** (○) to conscious rats ($n = 3$).

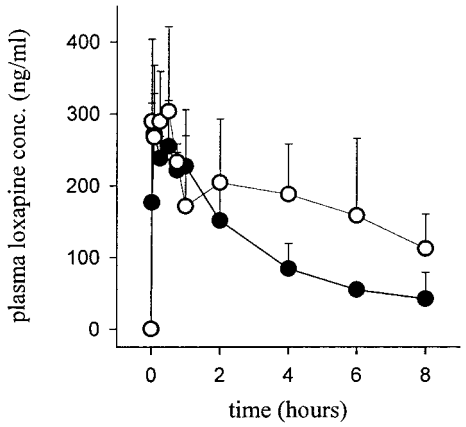


**Figure 4**—Plot of mean (±SD) plasma concentration versus time profiles of **6** following equimolar (30.5 $\mu$mol/kg) im injections of **6** (●) or **6-P** (○) to conscious rats ($n = 3$).

polysorbate 80), the ability of **6-P** to deliver **6** following intramuscular injection in an aqueous vehicle was investigated. Figure 4 illustrates the plasma concentration versus time profile following equimolar (30.5 $\mu$mol/kg) intramuscular injections of **6** and **6-P**. The mean AUCs of **6** after dosing with **6** and **6-P** were not significantly different ($\alpha = 0.05$) and the apparent bioavailability of **6** following **6-P** administration was found to be 1.27. Plasma levels of **6-P** were below the limit of detection (80 ng/mL) for all blood samples collected. Following intramuscular injection of **6-P**, a large fraction of the dose was immediately converted to **6**, which led to high plasma levels of **6** within 5 min of dosing. The **6-P** injections also resulted in some apparent elevations in **6** plasma levels 2−6 h postdosing; however, these apparent elevations are not statistically significant. The advantage of intramuscular administration of **6-P** in a purely aqueous formulation relative to **6** in its cosolvent system would be the potential avoidance of the tissue damage that is often associated when injecting organic cosolvents intramuscularly.[9,14]

**Evaluation of 7-P**—Figure 5 illustrates the plasma level versus time profiles of **7** following equimolar (6.2 $\mu$mol/kg) intravenous infusions of **7** and **7-P** to rats. There was no significant difference ($\alpha = 0.05$) between mean AUCs of **7** resulting from dosing with **7** and **7-P**. The apparent bioavailability of **7** following **7-P** administration was found to be 0.91.

To examine the potential for enzyme saturation, a **7-P** iv dose of 18.6 $\mu$mol/kg ($n = 3$) was given to the rats and compared to a 6.2 $\mu$mol/kg dose. This comparison is shown

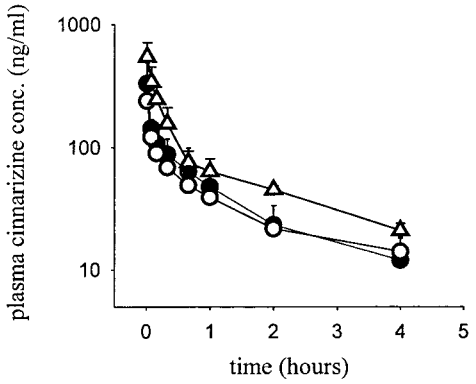


**Figure 5**—Semilog plot of mean (±SD) plasma concentration versus time profiles of **7** following equimolar (6.2 $\mu$mol/kg) 3 min iv infusions of **7** (●) or **7-P** (○) or 18.6 $\mu$mol/kg of **7-P** (△) to conscious rats ($n = 3$).

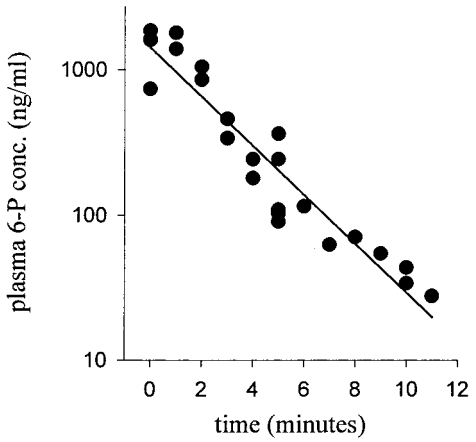


**Figure 6**—Semilog plot of plasma concentration of **7-P** as a function of time following a 3 min iv infusions of 6.2 $\mu$mol/kg of **7-P** (●) to conscious rats ($n = 4$).

in Figure 5. The mean AUC from the 18.6 $\mu$mol/kg dose (Table 1) was not statistically different from that for the 6.2 $\mu$mol/kg dose after dose correction. Any saturation that may have occurred through tripling of the dose of **7-P** could not be detected through AUC comparisons. As a result of limited quantities of prodrug, this experiment was not repeated with **6-P**.

The concentration versus time profile of **7-P** in plasma is shown in Figure 6. This plot contains data from a rat dosed with 6.2 $\mu$mol/kg of **7-P** for which blood samples were taken continuously for the first 10 min postinfusion to obtain a more complete profile. The disappearance of **7-P** from plasma was extremely rapid, and the half-life was estimated to be 2 min.

## Conclusion

*N*-Phosphonooxymethyl derivatives have been evaluated as prodrugs for increasing the aqueous solubility of drugs containing a tertiary amine functional group. Previous reports have demonstrated the synthetic feasibility, improved water solubility, good chemical stability, and ability to revert to parent drug in vitro in the presence of alkaline phosphatase.[1,2] To prepare injectable solutions of cinnarizine and loxapine required additions of complexing agent and cosolvents, respectively. Each of the prodrugs, however, was easily formulated in water near physiological pH. Following iv administration to rats and dogs, the prodrugs were rapidly and quantitatively reverted to parent drug. In addition, the loxapine prodrug was able to deliver parent drug by the im route.

Saturation of the enzyme systems responsible for prodrug to drug reversion was not evident following iv dosing of cinnarizine prodrug to rats when the prodrug dose was tripled from 6.2 to 18.6 $\mu$mol/kg. Evaluations of the linearity at higher doses was not possible because of limited prodrug quantities.

The in vivo hydrolysis of the prodrugs was remarkably fast considering the relatively slow in vitro hydrolysis in the prescence of alkaline phosphatase and in blood (see Experimental Section). One could speculate that enzymes contained in blood alone (at their respective concentrations) are not sufficient to explain the rapid hydrolysis found when the prodrugs were administered in vivo. It could be that a significant fraction of the enzymes responsible are membrane-associated and/or organ-specific. Additionally, contribution of enzymes other than alkaline phosphatase may significantly contribute to the in vivo hydrolysis.

As is shown in Scheme 1, prodrug reversion generates a mole equiv of formaldehyde and inorganic phosphate. Although formaldehyde has some known toxicity that may preclude long term, repetitive dosing with these prodrugs, the small amount of formaldehyde released should not present any significant problems. In fact, most drugs that undergo N- or O-demethylation will release formaldehyde as a metabolic product, and many of these drugs are clinically approved and minimally toxic.

In conclusion, the described prodrug approach appears to be a promising technique for safely overcoming the solubility limitations observed with tertiary amine containing drugs.

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## Acknowledgments


The authors thank Ms. Toni Bloodworth and Dr. Glenn Edwards for their assistance in these studies, the American Foundation for Pharmaceutical Education Fellowship, and the National Cancer Institute Training Grant for financial support. Additional support was provided by the Takeru Higuchi/Nigel Manning Intersearch program, Kansas University Endowment Association, the Center for Drug Delivery Research, and Kansas Technology Corporation through the Centers of Excellence program.

JS980382V