Hydrophobic Ion Pairing of Isoniazid Using a Prodrug Approach

HUIYU ZHOU,[1] CORINNE LENGSFELD,[2] DAVID J. CLAFFEY,[1] JAMES A. RUTH,[1] BROOKS HYBERTSON,[4] THEODORE W. RANDOLPH,[3] KA-YUN NG,[1] MARK C. MANNING[1]

[1]Department of Pharmaceutical Sciences, University of Colorado Health Sciences Center, Campus Box C238, 4200 E. Ninth St., Denver, Colorado

[2]Department of Engineering, University of Denver, Denver, Colorado

[3]Department of Chemical Engineering, University of Colorado at Boulder, Boulder, Colorado

[4]Webb-Waring Institute for Cancer, Aging, and Antioxidant Research and School of Medicine, University of Colorado Health Sciences Center, Denver, Colorado

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ABSTRACT: Inhalation therapy for infectious lung diseases, such as tuberculosis, is currently being explored, with microspheres being used to target alveolar macrophages. One method of drug encapsulation into polymeric microspheres to form hydrophobic ion-paired (HIP) complexes, and then coprecipitate the complex and polymer using supercritical fluid methodology. For the potent antituberculosis drug, isoniazid (isonicotinic acid hydrazide, INH), to be used in this fashion, it was modified into an ionizable form suitable for HIP. The charged prodrug, sodium isoniazid methanesulfonate (Na–INHMS), was then ion paired with hydrophobic cations, such as alkyltrimethylammonium or tetraalkylammonium. The logarithms of the apparent partition coefficients (log P') of various HIP complexes of INHMS display a roughly linear relationship with the numbers of carbon atoms in the organic counterions. The water solubility of the tetraheptylammonium–INHMS complex is about 220-fold lower than that of Na–INHMS, while the solubility in dichloromethane exceeds 10 mg/mL, which is sufficient for microencapsulation of the drug into poly(lactide) microspheres. The actual logarithm of the dichloromethane/water partition coefficient (log P) for tetraheptylammonium–INHMS is 1.55, compared to a value of -1.8 for the sodium salt of INHMS. The dissolution kinetics of the tetraheptylammonium–INHMS complex in 0.9% aqueous solutions of NaCl was also investigated. Dissolution of tetraheptylammonium–INHMS exhibited a first-order time constant of about 0.28 min^{-1}, followed by a slower reverse ion exchange process to form Na–INHMS. The half-life of this HIP complex is on the order of 30 min, making the enhanced transport of the drug across biological barriers possible. This work represents the first use of a prodrug approach to introduce functionality that would allow HIP complex formation for a neutral molecule.
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Keywords: hydrophobic ion pairing; prodrugs; isoniazid

INTRODUCTION

Inhalation therapy for infectious lung diseases is currently being explored,[1] with microspheres being used to target alveolar macrophages.[2] One

Correspondence to: Mark C. Manning (Telephone: 303-315-6162; Fax: 303-315-6281; E-mail: Mark.Manning@uchsc.edu)

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method we developed for drug encapsulation into polymeric microspheres is to form hydrophobic ion-paired complexes, and then coprecipitate the complex and polymer using supercritical fluid methodology termed precipitation with a compressed antisolvent (PCA).[3] Hydrophobic ion pairing (HIP) is a technique that increases the hydrophobicity of molecules containing ionizable groups by stoichiometrically replacing polar counterions with more hydrophobic ones. This process has been used to solubilize ionic molecules in nonpolar solvents,[4–7] enhance the transport of proteins and DNA,[8,9] and increase the bioavailability of ionic drugs.[10–14]

INH is an important drug used for the treatment of tuberculosis (TB).[15] Due to noncompliance with current TB therapies,[16–23] there has been an effort to develop alternative drug delivery systems to provide sustained release of anti-TB drugs at the site of infection (e.g., the lungs).[1,24–30] Therefore, incorporation of anti-TB drugs, like INH, into microparticles that would be small enough to provide efficient pulmonary delivery, is desirable. However, because INH is an uncharged, hydrophilic molecule, it is not amenable to ion pairing and its solubility in organic solvents is insufficient to use it directly in the PCA process. Therefore, to increase the solubility of INH in nonpolar solvents and to allow the use of the HIP and PCA processes, an ionizable prodrug of INH, Na–INHMS, was synthesized. Although HIP has been described in many systems,[4,5,10,11] this represents the first example of using a prodrug approach to introduce functionality that would allow ion pairing to take place, with the aim of subsequent encapsulation into microspheres for inhalation therapy.

MATERIALS AND METHODS

Materials

INH, dodecyltrimethylammonium bromide, tetradecyltrimethylammonium bromide, hexadecyltrimethylammonium bromide, tetraethylammonium bromide, tetraoctylammonium bromide were obtained from Sigma Chemical Co. (St. Louis, MO). Tetrabutylammonium bromide, tetrapentylammonium bromide, tetraheptylammonium bromide were purchased from Fluka (Switzerland). Dichloromethane was obtained from Sigma-Aldrich (Sigma Chemical Co., St Louis, MO and Aldrich Chemical Co., Milwaukee, WI). Ethyl alcohol (denatured) was from Aldrich (Milwaukee, WI). 2-Propanol was from Fisher Scientific (Fair Lawn, NJ). Silica gel ("Flash," 32–63 μm, 60 Å) was obtained from Scientific Adsorbent Inc. (Atlanta, GA). Medical grade compressed nitrogen was from General Air Service & Supply (Denver, CO).

Synthesis and Characterization of Na–INHMS

Briefly, Na–INHMS was synthesized using the procedure previously described by Logemann.[31] An aqueous (100 mL) solution of sodium bisulfite (52 g, 0.5 mol) was added to 0.5 mol of isoniazid (68.5 g) in 38 mL of 39% formaldehyde. The mixture was then heated at 100°C for 8 h and subsequently evaporated under reduced pressure to give a yellow solid. The yellow solid was dissolved in ethanol/water mixture. Recrystallization from the mixture gave Na–INHMS as a white solid. The purity of the derivative was characterized by both ^{1}H- and ^{13}C-NMR. ^{1}H-NMR (D_2O) spectrum of Na–INHMS: δ 3.9 (s, 2H), 7.5 (d, 2H, $J = 5.6$ Hz), 8.5 (d, 2H, $J = 5.56$ Hz). ^{13}C-NMR (D_2O) spectrum of Na–INHMS: δ 66.3, 121.8, 121.8, 140.6, 140.6, 149.6, 149.7, 166.9.

Determination of Extinction Coefficient of INHMS in Water

Approximately 78 mg of Na–INHMS powder was accurately weighed into a 50-mL beaker and dissolved in 20 mL of double distilled deionized water (DDW). The solution was transferred into a 100-mL volumetric flask. The beaker was rinsed with approximately 10 mL of DDW for three times, and all the liquid was transferred into the flask and diluted to volume with additional DDW. A series of aliquots (0.1, 0.2, 0.4, 0.6, 0.8, and 1 mL) of this stock solution were diluted to 10.0 mL with DDW and analyzed by UV spectrophotometry. The absorbance was measured at 262 ± 1 nm. A plot of absorbance versus drug concentration was linear within the range of 7.82–78.2 μg/mL ($r^2 = 0.99$). The extinction coefficient was determined to be $3.16 \times 10^3\ M^{-1} \cdot cm^{-1}$.

Determination of the Extinction Coefficient of INHMS in Dichloromethane

Approximately 175 mg of tetraheptylammonium–INHMS complexes were accurately weighed and dissolved into 10 mL of dichloromethane. A 0.01-mL volume of this solution was then diluted to 1 mL with dichloromethane and analyzed by

UV spectrophotometry. The absorbance was measured at 262 ± 1 nm. The extinction coefficient was calculated to be $(2.43 \pm 0.02) \times 10^3\ M^{-1} \cdot cm^{-1}$.

Formation and Partitioning of HIP–INHMS

Various HIP–INHMS were formed and extracted into organic solvents using the conventional flask-shake partitioning procedure.[32] Na–INHMS was first dissolved in DDW to make a 1 mM solution. To 2 mL of this solution, 2 mL of dichloromethane containing an organic ammonium salt at a concentration of 1 mM was added. The mixture was mixed by vortexing for 10 min and then was placed on a laboratory rocker (Enprotech, Integrated Separation Systems) for 2.5 h at approximately 18 cycles/min to ensure full interaction between the drug and the organic cations. The aqueous and the organic phases were then separated by centrifugation at 2000 rpm for 10 min. The drug concentrations in the aqueous and the organic layer were measured using UV spectrophotometry at 262 ± 1 nm. For the alkyltrimethylammonium complexes, the mass balance was quantitative. In the cases of the tetraalkylammonium salts, the recoveries ranged from 95–107%. Because this measurement did not distinguish between the different forms of INHMS (Na–INHMS, HIP–INHMS), the apparent partition coefficient (P') is reported as the value observed by direct determination of the total INHMS concentration in each phase (eq. 1) and cannot be considered a true partition coefficient.

$$P' = \frac{[S^+ \cdot D^-]_{org} + [Na^+ \cdot D^-]_{org}}{[S^+ \cdot D^-]_{aq} + [Na^+ \cdot D^-]_{aq}} \quad (1)$$

Here, D^- is the drug anion, which interacts with an organic counterion (S^+) to form the ion pair ($S^+ \cdot D^-$).

Isolation and Purification of the Tetraheptylammonium–INHMS Complex

To generate tetraheptylammonium–INHMS complexes for further characterization, the partitioning experiment described above was scaled up. The concentration of Na–INHMS and tetraheptylammonium bromide solutions was raised to 50 mM, and the volumes of both phases were increased to 10 mL. After phase separation, the organic layer was collected, dried under nitrogen and redissolved in a solvent consisting of 60% 2-propanol and 40% ethanol. To purify the tetraheptylammonium–INHMS complex, flash chromatography utilizing the Aldrich® flash-chromatography assembly with frit & System 45® connections (Sigma-Aldrich, Inc.) was used. Briefly, the "flash" silica gel was packed into the column to a height of 15–22 cm. The column was then flushed with a solvent consisting of 60% 2-propanol and 40% ethanol. After this initial equilibration process, samples were applied to the column as a 20–30% solution. Medical grade compressed nitrogen was applied to the column to maintain a flow rate of 1.5–2.5 mL/min. The eluents were collected as 5-mL aliquots and were analyzed for drug content and purity using UV spectrophotometry and thin layer chromatography. Aliquots containing the purified ion-paired complex was then dried using a SpeedVac dryer and saved for subsequent experimental use.

Measurement of the (True) Dichloromethane/Water Partition Coefficient of the Tetraheptylammonium–INHMS Complex

Two milliliters of dichloromethane containing 5 mM of the tetraheptylammonium–INHMS complex was mixed with 2 mL of DDW by vortexing for 5 min. The mixture was then centrifuged at 2000 rpm for 10 min. As no salt is present, there is no need to wait for equilibration between charged species, and this time has been found to be sufficient to allow partitioning of HIP complexes into two immiscible solvents.[2] At the end of the centrifugation, the organic layer was collected and analyzed for drug content by UV spectrophotometry. The drug content in the aqueous phase was determined by subtracting the drug content in the organic phase from the total drug content. The dichloromethane/water partition coefficient (P) was calculated according to eq. 2.

$$P = \frac{[S^+ \cdot D^-]_{org}}{[S^+ \cdot D^-]_{aq}} \quad (2)$$

In this case, INHMS exists only in the ion-paired form, so P represents the true partition coefficient for the complex.

Measurement of the Aqueous Solubility of Na–INHMS and the Tetraheptylammonium–INHMS Complex

Approximately 1000 mg of Na–INHMS or 250 mg of tetraheptylammonium–INHMS complexes were placed in a 20-mL glass vial (27×57 mm). Five milliliters of DDW were then added to the vial and the mixture was stirred at a speed of

approximately 300 rpm for 24 h at room temperature. At the end of the stirring, the supernatant was collected for determination of the drug concentration using UV spectrophotometry. To remove any particulates of the undissolved complex, supernatant was subjected to filtration using 0.2 μm pore size syringe filters.

Study of the Dissolution Kinetics of the Tetraheptylammonium–INHMS Complex

The purified tetraheptylammonium–INHMS complex is an extremely viscous semifluid. To maintain the amount as well as the surface area of the samples consistent, 2 mL of tetraheptylammonium–INHMS solution (200 mM) in dichloromethane was placed in a 20-mL glass vial (27×57 mm) and then allowed to evaporate to dryness in the hood. A film of tetraheptylammonium–INHMS would form on the bottom of the vial. Five milliliters of DDW or sodium chloride solution (0.9%) were then added to the vial. The solution was stirred at a speed of approximately 300 rpm. At designated time points, 0.1-mL aliquots were removed, filtered using a 0.2 μm pore size syringe filter, and assayed for drug content using UV spectrophotometry.

RESULTS AND DISCUSSION

The synthesis of INHMS proceeded using a modification of Logemann.[31] This allowed the incorporation of a readily ionizable group onto the INH pharmacophore. However, unless the group can be readily removed *in vivo*, it will not function as a true prodrug. Previously, INHMS and INH had been shown to have equal anti-TB activity both *in vivo* and *in vitro*.[33] Furthermore, Hsu and Ho demonstrated that INHMS was rapidly converted into INH and acetyl-INH upon administration to rabbits, suggesting the anti-TB activity of INHMS comes from its active metabolite, INH.[34] Thus, INHMS does appear to act as a prodrug of INH. Because INHMS is fully ionized under physiological conditions, it can be readily ion paired with hydrophobic counterions, with the ultimate goal of encapsulation into polymeric microspheres using PCA.

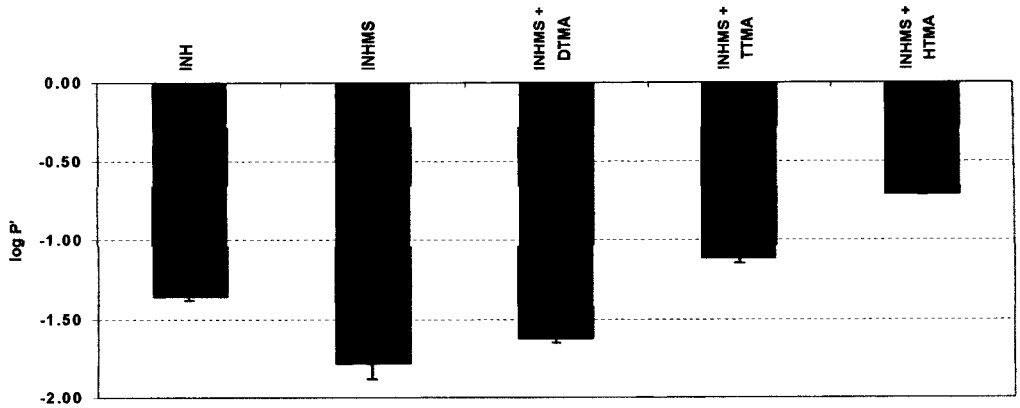
Formation and Partitioning of HIP–INHMS

In the partitioning experiments, HIP–INHMS were formed and then distributed between water and dichloromethane. The partitioning of HIP–INHMS ($S^+ \cdot D^-$) between the aqueous and organic phases can be characterized by the (true) partition coefficient (P) (eq. 2). However, in this study no attempt was made to distinguish between $Na^+ \cdot D^-$ and $S^+ \cdot D^-$ in solutions. Therefore, the partitioning behavior of the drug is reported as the apparent dichloromethane/water partition coefficient of the ion pair (P') (eq. 1). These are termed apparent partition coefficients, as no distinction is made to the nature of the counterion for the drug molecule in each phase. In each phase, the drug could be present either as the HIP complex or as the sodium salt.

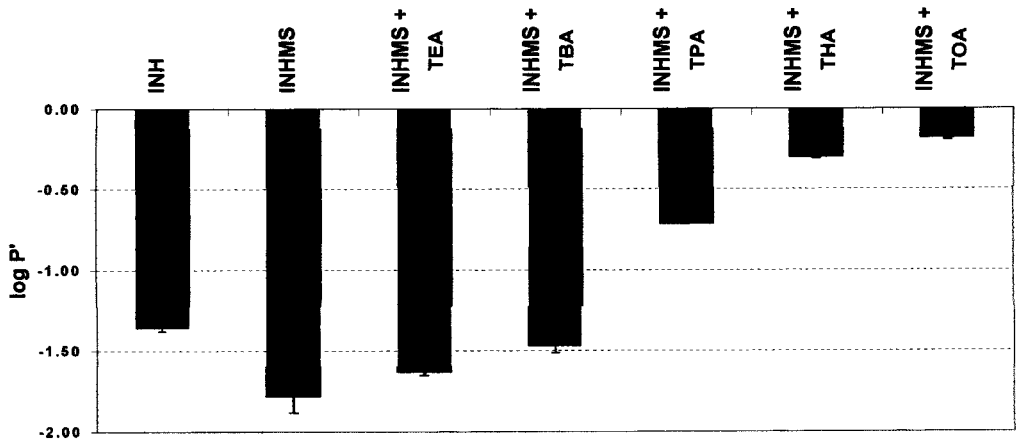
Hydrophobic counterions except dodecyltrimethylammonium increased the log P' of INHMS (Figure 1). In contrast, no surfactant-facilitated phase transfer of isoniazid was observed (data not shown), demonstrating the importance of the added negative charge on the isoniazid derivative. When log P' is very low, the errors in the measurement may be large, and may explain the slightly decreased log P' by dodecyltrimethylammonium compared to Na–INHMS alone. Three complexes—tetrapentylammonium–INHMS, tetraheptylammonium–INHMS, and tetraoctylammonium–INHMS—exhibited greater log P' than INH, suggesting these complexes are more hydrophobic than the parent drug. Note that the partition coefficients were measured in a dichloromethane/water, rather than the traditional octanol–water system. First, this is the most relevant solvent, as these HIP complexes must be solubilized in chlorocarbons to use PCA with poly(lactides) and poly(glycolides). Second, the amount of water present in 1-octanol after partitioning is significant (~1.7 M), and this might give an artificially low log P' value for such hydrophobic complexes. Finally, octanol–water measurements are most relevant when examining permeability across biological barriers, and this was not the goal of this study.

The partitioning of a HIP complex between an aqueous and an organic phase depends on its intrinsic hydrophobicity as well as the equilibrium constant for the ion-pair formation and the equilibrium constants for dissociation of all species in both phases.[4]

Two groups of cationic surfactants (tetraalkylammonium and trimethylalkylammonium) were examined for their capability of transferring INHMS to dichloromethane after the HIP complex formation. In general, within a particular homologous series, counterions with longer



(A)



(B)

Figure 1. Extraction of HIP–INHMS by dichloromethane. INH, isoniazid; INHMS, isoniazid methanesulfonate; DTMA, dodecyltrimethylammonium; TTMA, tetradecyltrimethylammonium; HTMA, hexadecyltrimethylammonium; TEA, teraethylammonium; TBA, tetrabutylammonium; TPA, tetrapentylammonium; THA, tetraheptylammonium; TOA, tetraoctylammonium. The values are the average ± standard deviation; $n = 3$.

hydrocarbon chains results in a larger P' for the HIP complex. Because the length of alkyl chains influences the size as well as the hydrophobicity of the organic cation, the relationship between the apparent partition coefficients of HIP–INHMS and the size/hydrophobicity of the organic cations was examined (Figure 2). The number of carbon atoms in the cation was used as an estimation of the relative size of the cation. Overall, the results indicate that log P' of HIP–INHMS follows a roughly linear relationship with respect to the number of carbon atoms in the organic counterions.

This type of behavior has been observed previously for HIP complexes. For example, Adjei et al. reported in a study on alkylsulfonate complexes with prolide that, for alkylsulfonates with short chains (C_6–C_{10}), there was a linear dependence of log P on the chain length.[35] Takács-Novák and Szász also reported a linear correlation between log P' of the ion pair and the size of the counterion, which was expressed as the solvent (water)-accessible surface area (SASA).[36] Using either the true partition coefficient (log P) of the pure ion pair[35] or the apparent partition coefficient (log P') values at 1:50 molar ratio

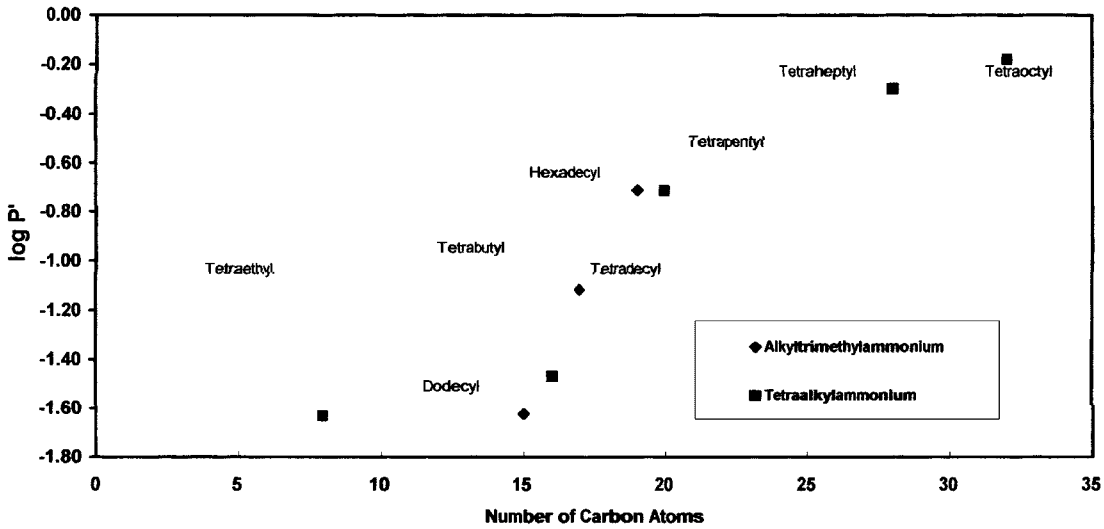


Figure 2. Relationship between the apparent partition coefficient (P') of HIP–INHMS and the number of carbon atoms in the organic counterions.

between the drug and the organic counter (diminishing the ion-pair dissociation by the excess organic counterions), the results are similar.[36] In our study, log P' of HIP complexes was measured at a 1:1 molar ratio between the drug and the organic counterion. Therefore, it appears that the presence of excess counterion does not greatly affect the relationship between the hydrophobicity of the species and the apparent partition coefficient. Together, these results indicate that increasing the size and overall hydrophobicity of the organic counterion produces a HIP complex that is more able to partition into a lipophilic phase than the parent compound, and that the relationship between counterion properties and the partitioning behavior is relatively well defined.

Hydrophobicity of the Tetraheptylammonium–INHMS Complex

Because the tetraheptylammonium–INHMS complex is readily extracted by dichloromethane, it can be easily produced on a larger scale, allowing for a more accurate measurement of the partition coefficient of a HIP complex of INHMS. Because the starting material is the intact, purified HIP complex and no salt is present, this value should represent the true dichloromethane–water partition coefficient.

The large P value suggests that appreciable solubility in dichloromethane should be obtained for this complex (Figure 3A). Ion pairing with tetraheptylammonium also increased the logarithm of the (true) water/dichloromethane partition coefficient (log P) from -1.8 to 1.55. The approximate 1700-fold increase in the actual partition coefficient is comparable to the increases reported for other HIP complexes.[3,6,7] The concentration of the tetraheptylammonium–INHMS complex in dichloromethane after direct partitioning approached 11 mg/mL, which is more than sufficient to allow subsequent incorporation in poly(L-lactide) microspheres using an antisolvent process.[12] Similarly, compared to that of Na–INHMS, the aqueous solubility of the tetraheptylammonium–INHMS complex decreased by about 220-fold (Figure 3B).

Dissolution Kinetics of the INHMS–Tetraheptylammonium Complex

An *in vitro* dissolution study was performed on the tetraheptylammonium–INHMS complex. One would expect that HIP complexes display biphasic kinetics when dissolved in an aqueous solution containing salts, with dissolution followed by dissociation of the HIP complexes.[37] However, little is known about the relative rates of these two processes for HIP complexes. After dissolution, dissociation of the complex is driven by ion exchange, and the drug concentration should increase, as the nonion paired drug is much more soluble in water than the corresponding HIP complex. The dissolution and dissociation behavior of HIP complexes can be determined by kinetic modeling. The first rate constant, k_1,

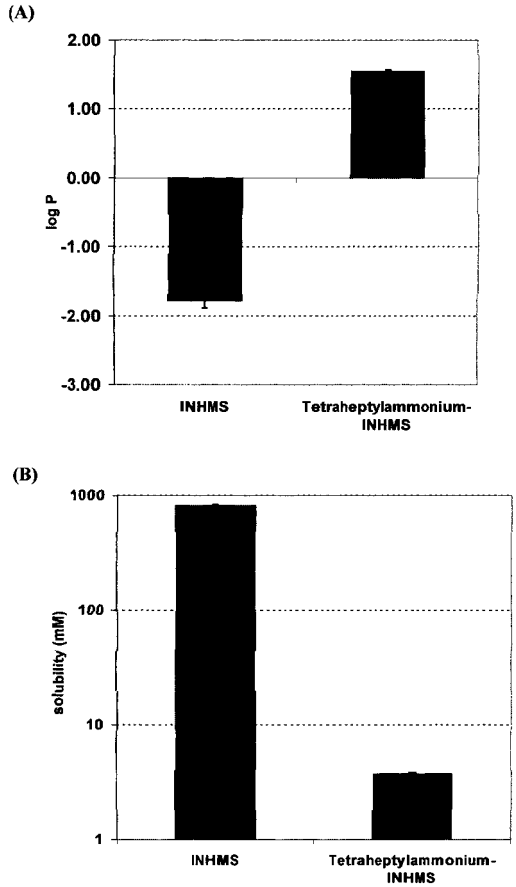


Figure 3. Effect of ion pairing with tetraheptylammonium on the water/dichloromethane partition coefficient (P) (A) and the aqueous solubility of INHMS (B). The values are the average ± standard deviation; $n = 3$.

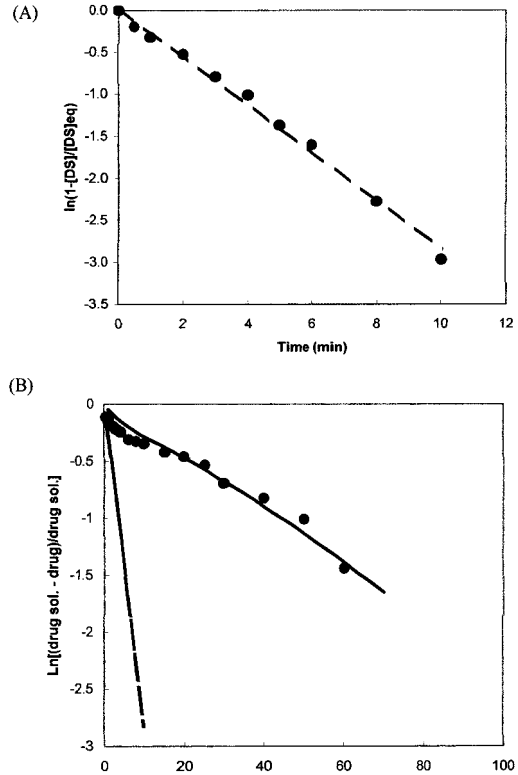


Figure 4. Plots of the ln(1-fraction released) versus time for the tetraheptylammonium–INHMS complex in DDW (A) and 0.9% sodium chloride solution (B). (A) Concentrations of the dissolved tetraheptylammonium–INHMS complex ([DS]), normalized by its equilibrium solubility in DDW ($[DS]_{eq}$), are plotted on a log scale versus time. The resulting slope, k_1, is the observed first-order rate constant for the HIP complex dissolution in DDW. (B) Concentrations of the dissolved INHMS ([D]), normalized by its equilibrium solubility in 0.9% sodium chloride solution ($[D]_{eq}$), are plotted on a log scale versus time. The dashed line indicates a simple model with a single dissolution rate; the solid line indicates a full model including reverse ion pairing.

describes the dissolution of the solid complex into liquid media. The other two rate constants, k_{2f} and k_{2r}, describes the rate of reverse ion pairing and ion pairing, respectively. It is important to note that the dissolution process (eq. 3), has been modeled as a kinetic phenomenon. As it will be dependent on stirring rates and other factors, it should be considered a relative value. Conversely, the ion exchange process has both a forward and reverse component, making it a true thermodynamic equilibrium. These factors were included in derivation of the model.[37] Figures 4 and 5 show dissolution/dissociation profiles of the tetraheptylammonium–INHMS complex in DDW and sodium chloride solutions. In DDW, only dissolution is possible, and the drug concentration plateaus at the solubility limit of the HIP complex (Figure 5).

$$(S^+ \cdot D^-)_{solid} \xrightarrow{k_1} (S^+ \cdot D^-)_{aq} \quad (3)$$

$$(S^+ \cdot D^-)_{aq} + NaCl_{aq} \underset{k_{2r}}{\overset{k_{2f}}{\Leftrightarrow}} (Na^+ \cdot D^-)_{aq} + (S^+ \cdot Cl^-)_{aq} \quad (4)$$

The dissolution behavior is well modeled by a single first order rate constant k_1, which was found to be 0.28 min^{-1} (Figure 4A and Table 1). This corresponds to a half-life ($t_{1/2}$) for dissolution of 2.5 min. This observed rate constant can be influenced by a number of factors, for example, the amount of samples, the sample–water surface

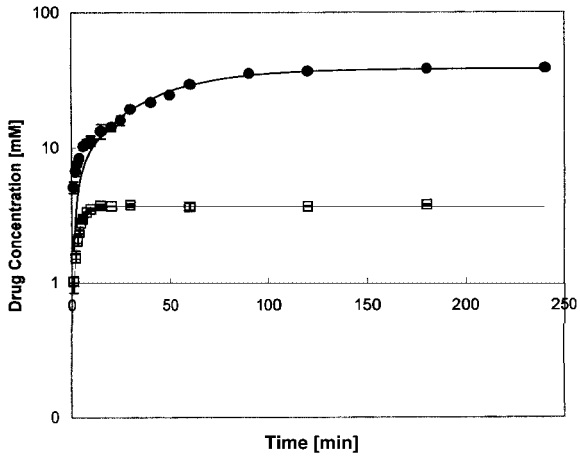


Figure 5. Dissolution profiles of the tetraheptylammonium–INHMS complex in DDW (□) and 0.9% sodium chloride solution (●) with profiles predicted from the model. For the measured concentrations, values are the average ± standard deviation; $n = 3$. For the model prediction, the estimated standard error is 2.12 mM.

area, and the mixing conditions, some of which were not precisely controlled or measured. Therefore, k_1 cannot be considered an intrinsic property of the complex, in contrast to k_{2f} and k_{2r}.

In normal saline, there are two distinct types of decays observed: one similar to the dissolution curve observed in pure water, and the other somewhat slower (Figures 4B and 5). Presumably, the first is due to dissolution, again with a half-life near 2.5 min. The second phase is slower, and must be due to reverse ion pairing with the sodium ions in solution. Table 1 lists the kinetic rate constants for the decay behaviors as well as the saline solubility of the HIP complex and total drug in 0.9% sodium chloride solution. They are derived based on the model built by Randolph and coworkers.[37] Figure 5 shows the dissolution profiles predicted by this model. The half-life for the second decay process appears to be about 25 min, with about 200 min being required to approach saturation under these conditions. The predicted values did not match data points at early time points very well. Because the drug concentrations at the initial stage were very low, the poor agreement is probably due to larger errors in making small measurements.

Detergent-enabled transport of proteins and nucleic acids through hydrophobic solvents by ion pairing has been shown in previous work.[8,9] In addition, formation of HIP complexes appears to increase bioavailability of ionic drugs.[10–14] All of this previous work suggests that the dissociation rates of HIP complexes are relatively slow and affected by the nature of the organic counterions used,[37] but none of these studies addressed this issue directly. This study provides a detailed description of not only the partitioning behavior of HIP complexes of INHMS, but also their propensity to dissociate in isotonic solutions.

CONCLUSIONS

A prodrug approach was employed to introduce a charged moiety into a neutral molecule, thereby allowing a hydrophobic ion-paired complex to be formed. As a result, the hydrophobicity of the prodrug is significantly enhanced, resulting in a 220-fold decrease in the water solubility and a 1700-fold increase in the dichloromethane/water partition coefficient. The dissolution kinetics of the HIP complex in an aqueous electrolyte solution exhibits a rapid dissolution phase followed by a slower reverse ion-pairing process. Although the half-life is on the order of 30 min, it is possible that the reverse ion pairing is still sufficiently slow to allow enhanced penetration of biological barriers by HIP complexes. Together, this study demonstrates that a prodrug approach can be taken to introduce a changed moiety into a neutral molecule, and that this compound can form stable HIP complexes. This now allows alternative methods of encapsulation into polymeric microspheres.

Table 1. Solubilities and Kinetic Rate Constants for Dissolution and Reverse Ion Exchange of the Tetraheptylammonium–INHMS Complex

Kinetic Rate Constants				Solubility (mM)		
k_1 (l/min)	k_{2f} (L/mol min)	k_{2r} (L/mol min)	Correlation Coefficient r^2	Measured Tetraheptylammonium–INHMS in DDW	Measured INHMS in 0.9% NaCl	Computed Tetraheptylammonium–INHMS in 0.9% NaCl
0.28	0.55	0.56	0.984	3.7	38.7	7.8

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