# A Mathematical Relationship for Hydromorphone Loading into Liposomes with Trans-Membrane Ammonium Sulfate Gradients

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**ABSTRACT:** We have studied the loading of the opioid hydromorphone into liposomes using ammonium sulfate gradients. Unlike other drugs loaded with this technique, hydromorphone is freely soluble as the sulfate salt, and, consequently, does not precipitate in the liposomes after loading. We have derived a mathematical relationship that can predict the extent of loading based on the ammonium ion content of the liposomes and the amount of drug added for loading. We have adapted and used the Berthelot indophenol assay to measure the amount of ammonium ions in the liposomes. Plots of the inverse of the fraction of hydromorphone loaded versus the amount of hydromorphone added are linear, and the slope should be the inverse of the amount of ammonium ions present in the liposomes. The inverse of the slopes obtained closely correspond to the amount of ammonium ions in the liposomes measured with the Berthelot indophenol assay. We also show that loading can be less than optimal under conditions where osmotically driven loss of ammonium ions or leakage of drug after loading may occur. © 2009 Wiley-Liss, Inc. and the American Pharmacists Association J Pharm Sci 99:2672–2680, 2010

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## INTRODUCTION

The use of ammonium sulfate gradients for the efficient loading of weakly basic drugs into liposomes was first developed for the loading of doxorubicin into liposomes.[1] Subsequently, this technique has been used for the loading of other weakly basic drugs into liposomes, including ciprofloxacin and bupivicaine.[2–5] Loading is achieved when ammonium sulfate is first captured in the liposomes, and subsequently eliminated from the extraliposomal space by dialysis or desalting. Elimination of ammonium sulfate from the extraliposomal space causes rapid efflux of ammonia from the liposomes, which is produced by the dissociation of ammonium ions to give ammonia and protons, thereby reducing the intraliposomal pH. The pH gradient can be as much as 3–4 pH units between the intraliposomal and extraliposomal compartment.[1] Upon addition of a weakly basic drug to the liposomes, highly efficient loading occurs through the influx of the free base and subsequent accumulation of the protonated form in the acidic intraliposomal compartment.

Opioids are chemical substances with morphine-like action through binding to opioid receptors in the central and peripheral nervous system, and the gastrointestinal tract.[6] They have long been used to treat acute and chronic pain. However, the rapid clearance of opioids by first pass metabolism in the liver, especially in species such as dogs, limits both the oral usage and the duration of the effect.[7] Although a larger dose will increase the duration of the effect, dose is limited by the side-effects of opioids, especially at peak drug concentration, which include respiratory depression, sedation, coma, and death.[6] Therefore, controlled release of opioids is a potential approach to overcoming the limited duration of effect, and we have recently documented the potential utility of liposome-encapsulated opioids for long-term management of pain in animals. The period of analgesia, which is about 2–4 h for an i.v. bolus injection, is increased when drug is administered subcutaneously in liposomes. The duration of serum concentration and analgesic effects can be extended to 24 h through the use of egg phosphatidylcholine/cholesterol liposomes.[8] The duration of serum concentration and analgesic effects is increased to 96 h through the use of dipalmitoylphosphatidylcholine/cholesterol

liposomes.[9] Initially, we used the dehydration–rehydration method of Kirby and Gregoriadis to prepare liposomes.[10] More recently, we have used ammonium sulfate gradients to achieve more efficient loading of DPPC/cholesterol liposomes. Interestingly, the period of analgesia is greatly increased in macaque monkeys by ammonium sulfate loading of oxymorphone to as much as 3 weeks.[11]

Ammonium sulfate gradient loading efficiency of oxymorphone is typically 30–40%,[11] while the efficiency of passive aqueous capture can be as low as 15% for hydromorphone.[9] In contrast, doxorubicin loading efficiency can be as high as 99%.[1] In order to learn more about the loading of opioids using ammonium sulfate gradients and how efficiency might be increased, we have studied the relationship between loading efficiency and the amount of drug added. We have developed a simple mathematical relationship capable of predicting the fraction of drug that will load into liposomes from the amount of ammonium ions trapped in the liposomes prior to loading, and from the amount of drug added to the liposomes during loading. As required for the relationship to be predictive, the saturating concentration of hydromorphone was found to exceed the concentration present in liposomes after loading.

## MATERIALS AND METHODS

Dipalmitoylphosphatidylcholine (DPPC), egg phosphatidylcholine (egg PC), and cholesterol were purchased from Avanti Polar Lipids (Alabaster, AL). Hydromorphone hydrochloride was purchased from PCCA (Houston, TX). Sephadex G 50 was purchased from Sigma-Aldrich (St. Louis, MO). All other reagents were reagent grade or better.

### Liposome Preparation

A mixture of phospholipid/cholesterol 1.6:1 (mol/mol) dissolved in chloroform was evaporated in a rotary evaporator, and suspended in *tert*-butanol at 80 μmol/mL phospholipid, and freeze-dried to produce a microporous lipid mixture. Multilamellar vesicles (MLV) were prepared by suspending the lipid mixture at 80 μmol/mL phospholipid in aqueous medium with vigorous shaking for 5 min. The lipid dispersion was allowed to stand at 25°C under argon for 1 h prior to subsequent steps.

Small unilamellar vesicles (SUV) were prepared by ultrasonication of egg PC/cholesterol MLV for 10 min at 25°C using a bath type sonicator (Laboratory Supplies, Inc., Hicksville, NY). The suspension was sedimented at 160,000 × g for 4 h in an ultracentrifuge to eliminate any residual larger liposomes, leaving a highly uniform SUV population in the supernatant.[12]

Large unilamellar vesicles (LUV) were prepared by extrusion of MLV six times through a 0.2 μm polycarbonate filter, using a stainless steel extrusion cell (Mico Instruments, Middleton, WI) at a pressure of 120 psi at 25°C.

### Size Distribution

Liposome size distribution was determined by dynamic light scattering (DLS), using a Nicomp Model 380 (Nicomp, Santa Barbara, CA).

### Formation of Ammonium Sulfate Gradient

For MLV, the unencapsulated ammonium sulfate was removed by sedimentation of the liposomes at 300 × g in a benchtop centrifuge, the pellet was washed twice by resuspending it in 0.9% (w/v) NaCl and sedimenting as before. For SUV and LUV, the unencapsulated ammonium sulfate was removed by dialysis two times for 12 h against 1 L 0.9% (w/v) NaCl at room temperature.

### Ammonia Measurement

Ammonia was measured using an adaptation of the Berthelot indophenol method described by Jaenicke[13] for measurement of nitrogenous materials. Liposomes were first extracted using the method of Bligh and Dyer.[14] Briefly, 20 μL liposome sample was mixed with 140 μL water, 400 μL MeOH, and 200 μL CHCl$_3$. Another 200 μL CHCl$_3$ was added to give a cloudy mixture. Addition of 200 μL 0.9% NaCl and centrifugation at 300 rpm for 10 min gave two separate clear phases. The lower CHCl$_3$ phase was aspirated out from under the upper phase, and the upper phase, which contained H$_2$O, MeOH, and (NH$_4$)$_2$SO$_4$, was transferred to large test tubes. After 0.4 mL 10 M sulfuric acid was added to each tube, the tubes were transferred to a 165°C hot block for 30 min to evaporate off the MeOH and water. The sulfuric acid was necessary to retain the ammonia. After cooling, 0.8 mL 5 M NaOH was added to each tube to neutralize the acid. After further cooling, 1 mL 2.125% (w/v) phenol, 0.0125% (w/v) sodium nitroprusside was added to all tubes with agitation. Finally, 0.4 mL 0.02 M sodium hypochlorite in 2.5 M NaOH was added with mixing. The solutions were allowed to stand at room temperature for exactly 20 min, and the absorbance at 578 nm was measured using a Hitachi-3000 UV/Vis spectrophotometer (Hitachi Instruments, San Jose CA). In every batch of samples, standards were included that contained 0, 0.1, or 0.2 μmol NH$_3$ (as ammonium sulfate). Standards were also subjected to extraction and evaporation.

### Liposome Loading With Hydromorphone HCl

A solution of hydromorphone hydrochloride was added to 0.5 mL of the liposome dispersion (20–80 mM phospholipids) after the creation of ammo-