# Effects of bile salts on propranolol distribution into liposomes studied by capillary electrophoresis

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## ARTICLE INFO

## ABSTRACT

The objective of this study was to study the effect of four different bile salts, cholate (C), deoxycholate (DC), taurocholate (TC), monoketocholate (MKC), on the membrane binding of a cationic model drug, propranolol, using capillary electrophoresis. The apparent distribution coefficient of propranolol in a buffer/liposome system, in the absence and presence of various concentrations of the bile salts, was measured using capillary electrophoresis frontal analysis. At bile salt concentrations which did not disrupt the liposomes, the bile salts increased the apparent distribution coefficient of propranolol in a concentration-dependent manner, to various extents (DC > C > TC > MKC). The mechanisms for these increases were inferred from studies of ion pairing between bile salts and propranolol using mobility shift affinity capillary electrophoresis and from zeta potential measurements. The bile salts ion-paired with propranolol to different extents as indicated by the estimated complexation constants ($K$ range: 30–58 $M^{-1}$). This was found to have a minor effect on the membrane distribution of propranolol only. The major effect is proposed to be due to the insertion of bile salt into the liposomal membranes leading to a more negatively charged membrane surface thereby providing stronger electrostatic interactions with the positively charged propranolol.

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## 1. Introduction

Bile salts are endogenous surfactants which have been studied widely as permeability enhancers to increase drug transport across various biological barriers such as buccal mucosa [1], intestine, skin [2], cornea [3] and blood–brain barrier [4]. They increase paracellular permeability at relatively high submicellar concentrations [5], whereas bile salt micelles are cytotoxic solubilizing cell membranes and remove membrane components [6]. At sub-lytic concentrations, bile salt monomers insert into cell membranes, the extent being determined by their lipophilicity [7]. The accumulation of bile salts in cell membranes not only changes the membrane composition but may also alter membrane biophysical properties such as membrane fluidity [8] which may increase passive diffusion of drugs across the membranes.

At physiological pH, bile salts are negatively charged in aqueous solution so that their incorporation into lipid bilayers increases the negative surface charge density. Since the interaction of ionized drugs with membranes may involve electrostatic interactions [9], membrane incorporated bile salts have the potential to affect the partitioning of ionized drugs. Moreover, bile salts are not only able to interact with membranes but can also interact with drug molecules [10,11]. Several studies have shown that bile salts form ion pairs with cationic compounds thereby increasing the apparent lipophilicity of the parent drugs and subsequently increasing drug absorption [12–14].

An anisotropic liposome/buffer system is considered as a more bio-relevant model to predict drug absorption, compared to the isotropic octanol/water system, particularly when electrostatic interactions between ionized drugs and membranes are significant [15]. Various techniques have been developed to determine buffer/liposome distribution coefficients: equilibrium dialysis [16], potentiometry [17], NMR [18] and second-derivative spectrophotometry [19]. CE-FA has been widely explored for the investigation of plasma protein binding and polyelectrolyte complexation and the data have generally been found to be in good agreement with results obtained using other techniques, such as equilibrium dialysis and ultrafiltration [20–22]. More recently, capillary electrophoresis frontal analysis (CE-FA) methods have been developed to study drug liposome interactions and to determine liposome/buffer distribution coefficients [23–25]. The compara-

tive studies available have demonstrated satisfactory agreement between affinity data obtained using equilibrium dialysis and CE-FA [26] and electrokinetic chromatography and CE-FA [27], respectively. Compared with techniques such as equilibrium dialysis, CE-FA is relatively fast, less labour intensive and requires small amounts of sample [26].

The aim of this study was to investigate the effects of four bile salts (Fig. 1(a)), cholate (C), deoxycholate (DC), taurocholate (TC), monoketocholate (MKC), which have been characterized as permeability enhancers using a 1,2-dipalmitoyl-$sn$-glycero-3-phosphocholine (DPPC) (Fig. 1(c)) monolayer model [28], on the distribution of propranolol (Fig. 1(b)) ($pK_a$ 9.5), a cationic model drug at physiological pH, into lipid bilayers. The same lipid as used in the monolayer studies, DPPC [28], was employed in the preparation of the unilamellar liposomes subject to investigation. A CE-FA method, suitable for determining the apparent liposome/buffer distribution coefficient of propranolol in the absence and presence of bile salts, was developed. The interactions between the bile salts, at submicellar concentrations, and propranolol were characterized by mobility shift affinity capillary electrophoresis (ACE) [29–33]. The results of this study should help in understanding the effects of bile salts on the distribution of cationic drugs into biological membranes and, consequently, the effect of bile salts on the passive diffusion of these drugs across biological barriers via the transcellular pathway.

## 2. Materials and methods

### 2.1. Materials

DPPC, HEPES, propranolol hydrochloride, Ringer's buffer (10 mM D-glucose; 0.5 mM MgCl$_2$; 0.45 mM KCl; 120 mM NaCl; 0.70 mM Na$_2$HPO$_4$; 1.5 mM NaH$_2$PO$_4$), C, DC and TC were purchased from Sigma–Aldrich (St. Louis, MO, USA). MKC, purity 96.5% with 3.1% cholate as the major impurity determined by HPLC and MS, was a gift from Professor Ksenija Kuhajda (University of Novi Sad, Serbia). All other chemicals and reagents were of at least analytical grade. Purified water prepared from a Milli-Q deionization unit (Millipore, Bedford, MA, USA) was used throughout.

### 2.2. Methods

#### 2.2.1. Preparation and characterization of liposomes
Unilamellar liposomes were prepared in a round-bottomed flask by dissolving 20 mg DPPC in chloroform. The organic solvent was removed by rotary evaporation and dried overnight under vacuum. The lipid film was hydrated with 5.0 mL Ringer's–HEPES buffer (Ringer's buffer containing 10 mM HEPES, pH 7.4) for 1 h at 55 °C. Upon hydration, the dispersion was left at room temperature to settle for 1 h. In order to obtain unilamellar liposomes, extrusion of the liposomal dispersion was performed 10 times through two stacked polycarbonate filters (Whatman International, UK) with pore size of 100 and 50 nm at 55 °C using an extruder (LipexBiomembranes, Vancouver, Canada) under nitrogen pressure. The concentration of DPPC in the liposome preparation was determined using the Stewart assay [34]. The liposome preparations were stored at 4 °C until use.

#### 2.2.2. Determination of particle size and zeta potential
Zetapotential ($\zeta$-potential) values and the size distribution of the DPPC liposomes were determined in Ringer's–HEPES buffer at pH 7.4 and 25.0 °C by dynamic light scattering (DLS) analysis using a Zetasizer ZS90 (Malvern Instruments Ltd., Malvern, UK). The lipid concentration was kept constant at 2 mM and the bile salt concentration was in the range 0–3 mM. Viscosity and refractive index of the dispersion medium (water) were taken as 1.02 cP and 1.330, respectively.

#### 2.2.3. CE-FA experiments
CE-FA was performed on a HP $^{3D}$CE instrument (Agilent Technologies, Waldbronn, Germany) equipped with a diode array detector (DAD). Uncoated fused silica capillaries, 50 µm id and length of 32.5 cm, with a length of 24.5 cm to the detector, were used in all experiments (Polymicro Technologies, Phoenix, AZ, USA). New capillaries were conditioned by flushing sequentially with 1 M NaOH, 0.1 M NaOH and Ringer's–HEPES buffer for 30 min each. The capillary was flushed daily with 1% SDS solution, 1 M NaOH and Ringer's–HEPES buffer for 5 min each before conducting experiments. Between runs, the capillary was flushed with 0.1 M NaOH and Ringer's–HEPES buffer for 2 min each. UV detection was performed at 214 nm. The applied voltage was +5 kV (∼50 µA) and samples were introduced by hydrodynamic injection (50 mbar for 20 s) unless otherwise reported. The temperature of the capillary cassette was set to 25 °C. Ringer's–HEPES buffer was used for all CE experiments and sample preparation. All samples were mixtures of 100 µM propranolol, bile salts at various concentrations and 2 mM DPPC liposomes. Standard samples were mixtures of 100 µM propranolol and bile salts without DPPC liposomes. All samples and standards were analyzed in triplicate. Standard samples were analyzed immediately before liposome-containing samples with the same total drug concentration.

#### 2.2.4. Calculation of distribution coefficient from CE-FA experiments
The propranolol concentration in the aqueous phase ($C_{aq}$) was calculated from the total drug concentration ($C_{total}$) and the plateau peak heights measured by CE-FA for the drug substance in the liposome-containing sample ($H_{sample}$) and in the standard solution ($H_{std}$) containing the same total drug concentration but without liposomes:

$$C_{aq} = \frac{H_{sample}}{H_{std}} C_{total} \tag{1}$$

The apparent membrane distribution coefficient was defined as:

$$D_{mem} = \frac{C_{mem}}{C_{aq}} \tag{2}$$

where $C_{mem}$ is the concentration of drug in the membrane (liposomal) phase. The drug concentration in the membrane phase was calculated by mass balance:

$$C_{mem} = \frac{C_{total}V_{total} - C_{aq}V_{aq}}{V_{mem}} \tag{3}$$

where $V_{total}$, $V_{aq}$ and $V_{mem}$ are the total sample volume, volume of the aqueous phase and volume of the membrane phase, respectively. $V_{mem}$ was calculated from the lipid concentrations determined using the Stewart assay assuming the density of the lipid membrane phase to be 1.00 g/mL [35,36]. The volume of the aqueous phase, $V_{aq}$, was calculated from the relationship:

$$V_{total} = V_{aq} + V_{mem} \tag{4}$$

Note that $V_{mem}$ was defined to include only the volume of the phospholipid and, thus, does not take into account the increase in volume due to incorporation of the bile salts into the liposomes. Experiments were conducted in triplicate. Mean and variance of a ratio of peak heights of liposome-containing sample ($H_{sample}$) and the standard solution ($H_{std}$) were calculated according to Taylor expansions [37].