Determination of the Aggregation Constants of Bile Salts by Capillary Electrophoresis

J. Gyimesi1* / Z. Szakács2 / M. Tarnai23 / E. Szőkö4

1Pharmavit Co., Lévai u. 5, H-2112 Veresegyház, Hungary
2Department of Inorganic and Analytical Chemistry, Loránd Eötvös University, Pázmány Péter sétány 1/A, 1117 Budapest, Hungary
3Present address: Graduate School of Environmental Earth Science, Hokkaido University, Sapporo 060, Japan
4Department of Pharmacodynamics, Semmelweis University of Medicine, Nagyvárad tér 4, 1089 Budapest, Hungary

Key Words
Capillary zone electrophoresis
Bile salts
Aggregation number
Aggregation equilibrium constant

Summary
Capillary electrophoresis has been investigated as a novel experimental method for determination of the aggregation constants of surfactants. The tendency of sodium cholate and sodium taurodeoxycholate to associate was studied in phosphate buffers of pH 8.0 and pH 7.0, respectively. Stepwise aggregation equilibria of bile salt monomers has been described in terms of mass-balance equations. The Offord equation was used to model the electrophoretic mobility of the bile salt associates, and the experimental mobility values could be fitted to the model. Interestingly, only even-membered aggregates—dimers and tetramers—besides the monomers were proposed from the results of the curve-fitting for both bile salts. The aggregation constants calculated were (in molar units): cholate log $K_{A2} = 1.37$, log $K_{A4} = 4.98$; taurodeoxycholate log $K_{A2} = 1.68$, log $K_{A4} = 6.46$. From these values, more pronounced aggregation of taurodeoxycholate starting at lower concentrations has been deduced, supporting the back-to-back association model of bile salts.

Introduction
Since Terabe’s pioneering work [1, 2], micellar electrokinetic chromatography (MEKC) has become a well established technique for the separation of uncharged hydrophobic and ionic compounds, closely related peptides, or complex mixtures [3]. The incorporation of chiral surfactants into the background electrolyte also offers the possibility of separating enantiomers [4]. Bile salts are the most widely used natural chiral surfactants. Most chiral separation studies focus on optimization of enantiomer resolution [5–9] and relatively little is known about the quantitative characteristics of the analyte–micelle interaction. The most widely accepted physicochemical model is the partitioning of the analyte between the aqueous and micellar phases [10]. To use this model for quantitative purposes the (critical micellar concentration) CMC of the surfactant must be accurately known under the CE separation conditions.

Although the aggregation properties of bile salts have been extensively investigated by various methods [11–15], the results of these studies are often contradictory, partly because of the inherent limitations of the experimental techniques used [11]. Even the existence of CMC values for some bile salts has been questioned and continuous build-up of aggregates has been proposed instead of a well-defined CMC value [12]. In accordance with the scatter observed in aggregation numbers, different models of bile salt aggregation have been proposed [12, 16, 17]. Aggregation constants for these compounds are not widely available in the literature [18, 19]. For analytical purposes, it is difficult to predict the correct aggregation parameters valid under the conditions of the capillary electrophoretic run. The problems associated with the CMC can be avoided if aggregation is treated as a stepwise equilibrium process [10]. Capillary electrophoresis has proved to be an efficient tool to for determination of different types of equilibrium constant–acidity constants [20, 21], metal ion–ligand complex formation constants [22], and binding constants in drug–cycloextrin [23], benzoate–cycloextrin [24], ligand–antibody [25], and polyelectrolyte–enzyme [26] systems.

In this study the applicability of CE for the determination of bile salt aggregation constants has been investigated. Bile salt samples of different concentration were analyzed by capillary zone electrophoresis (CZE) and model equations were derived to fit the experimental mobility values. Results for sodium cholate and sodium taurodeoxycholate are presented.
Theory

The stepwise aggregation of the bile salt monomer, b, leading to the formation of dimers, b₂, and trimers, b₃, can be described by the equilibria:

\[
2b \rightleftharpoons b_2 \quad K_{A2} = \frac{[b_2]}{[b]^2} \quad (1)
\]

\[
3b \rightleftharpoons b_3 \quad K_{A3} = \frac{[b_3]}{[b]^3} \quad (2)
\]

\[
nb \rightleftharpoons b_n \quad K_{A_n} = \frac{[b_n]}{[b]^n} \quad (3)
\]

where \( K_{A2} \) and \( K_{A3} \) are the overall aggregation constants and \( n \) is the maximum aggregation number in the concentration range studied. The injected sample zone, in which the total concentration of the monomer, \( Q \), is known, contains a mixture of the different associates \( b_i \). The total concentration of the bile salt aggregates, \( C_{\text{tot}} \), is obtained by summing the equilibrium concentration \( [b_i] \) of each associate:

\[
C_{\text{tot}} = [b] + [b_2] + \ldots + [b_n] = [b] + \sum_{i=2}^{n} [b_i] \quad (4)
\]

If the association equilibrium occurs during electrophoresis, a compound existing in several forms with different mobilities in equilibrium with each other will migrate as a uniform substance and only one peak is expected in the electropherogram [20, 25, 27, 28]. An average electrophoretic mobility, \( \mu_{\text{exp}} \), can, therefore, be calculated for the analyte:

\[
\mu_{\text{exp}} = \frac{[b]}{C_{\text{tot}}} \mu_b + \frac{[b_2]}{C_{\text{tot}}} \mu_{b_2} + \ldots + \frac{[b_n]}{C_{\text{tot}}} \mu_{b_n} = \frac{[b]}{C_{\text{tot}}} \mu_b + \sum_{i=2}^{n} \frac{[b_i]}{C_{\text{tot}}} \mu_{b_i} \quad (5)
\]

where \( \mu_b \) denotes the electrophoretic mobility of the bile salt monomer and \( \mu_{b_i} \) is the mobility of an aggregate. Substitution of Eqs (1)–(3) into Eq. (5) yields:

\[
\mu_{\text{exp}} = \frac{[b]}{C_{\text{tot}}} \mu_b + \sum_{i=2}^{n} \frac{K_{A_i}[b_i]}{C_{\text{tot}}} \mu_{b_i} \quad (6)
\]

In this equation only \( \mu_{\text{exp}} \) is an experimental quantity – the \( K_{A_i} \) aggregation constants and \( \mu_{b_i} \) mobilities are all unknown. Although, in principle, values of \( \mu_b \) and \( \mu_{b_n} \) can be estimated from electropherograms obtained from bile salts present at very low and very high concentrations, respectively, the aggregation constants cannot be evaluated without invoking a formal relationship between the values of the various \( \mu_{b_i} \) [28].

Two assumptions are made about the hydrodynamic properties of the associates.

(i) First, we assume that the hydrodynamic drag of the aggregates does not vary significantly over the concentration range studied. Hence, we can use the Offord equation to approximate the mobility of the aggregates. The applicability of this empirical relationship has been proved for peptides [29] and small globular proteins [30] and its potential application has also been considered in some binding studies [10, 25]. Applying the Offord equation to the bile salt aggregates gives:

\[
\mu_{\text{O}} = \frac{Z_i}{M_i^{2/3}} - \frac{Q_i}{M_i^{2/3}} \frac{\mu_{b_i}}{M_b^{2/3}} \quad (7)
\]

where \( Z_i \) and \( M_i \) are the charge and mass of the \( i \)th aggregate and \( Q_b \) and \( M_b \) are the effective charge and mass, respectively, of the monomer. \( Y \) is a proportionality constant, which can be combined with \( Z_i \) to obtain a formal charge \( Q_i \).

(ii) Our second assumption is that the charges on associates of increasing aggregation number are compensated to the same extent by an increasing number of counterions (Na⁺) [15, 31]. If so, the effective charge of the subsequent aggregates grows by the same effective monomer charge, \( Q_b \), in Eq. (7).

Combining Eqs (6) and (7) yields:

\[
\mu_{\text{exp}} = \frac{[b]}{C_{\text{tot}}} \cdot \frac{Q_b}{M_b^{2/3}} + \sum_{i=2}^{n} \frac{K_{A_i}[b_i]}{C_{\text{tot}}} \cdot \frac{\mu_{b_i}}{M_b^{2/3}} \quad (8)
\]

where only \( Q_b \) and the \( K_{A_i} \) aggregation constants are unknown, and can be calculated by non-linear curve-fitting methods.

Experimental

Chemicals

Sodium taurodeoxycholate (STDC) of analytical grade was purchased from Sigma–Aldrich (Budapest, Hungary) and cholic acid from Merck (Darmstadt, Germany). Both bile salts were used without further purification. Other reagents were of analytical grade (Sigma-Aldrich). Twice-distilled deionized water was used to prepare all solutions.

Capillary Electrophoresis Apparatus and Procedures

Studies were conducted with a Prince (Lauerlabs, Emmen, The Netherlands) apparatus equipped with an LTV detector operated at 210 nm. Uncoated fused-silica capillaries, 70 cm × 50 μm, 55 cm to detector, were purchased from Supelco. Axxi-Chrom 727 software (Axxiom Chromatography, Moorpark, CA, USA) was used for data collection. Before each set of measurements the capillary was flushed with 0.10 M NaOH for 5 min, then with water for 5 min, and finally with the running buffer for 10 min.

The running buffer for STDC samples was 66 mM sodium phosphate buffer (pH 7.0, ionic strength, \( I = 0.1 \) M); STDC samples (\( C_b = 0.5–48 \) mM) were prepared from aqueous STDC stock solution by dilution with the running buffer. The running buffer for cholic acid was 40 mM.