Binding of Low-Molecular-Weight Cationic Ligands to Chondroitin Sulfate as Studied by Capillary Electrophoresis Frontal Analysis

Jesper Østergaard*, Henrik Jensen, Susan W. Larsen and Claus Larsen

Abstract: The feasibility of capillary electrophoresis frontal analysis for the study of low-molecular-weight ligand-chondroitin sulfate interactions was investigated. The interaction of the 7 cationic ligands (lidocaine, propranolol, scopolamine, N-methyl scopolamine, N-butyl scopolamine, 2-propylisochinolinium, and methyl viologen) with the glycosaminoglycan chondroitin sulfate was investigated in 0.067 M phosphate buffer, pH 7.4, at 25°C. A frontal analysis method with a short analysis time, suitable for ranking ligands according to degree of complexation for chondroitin sulfate was developed. The double charged ligand methyl viologen possessed the highest affinity for chondroitin sulfate. Linear binding isotherms were obtained and apparent association constants were determined. Capillary electrophoresis frontal analysis may be an attractive tool for characterization of ligand-glycosaminoglycan interactions.

Keywords: Affinity capillary electrophoresis, chondroitin sulfate, complexation, frontal analysis, drug-glycosaminoglycan interactions.

INTRODUCTION

Glycosaminoglycans, such as chondroitin sulfate, play an important role in biomedical sciences and pharmaceutics. These high-molecular-weight structures are present in most tissues and body fluids. Small-molecule amphiphilic drug substances may interact with glycosaminoglycans. It is known that drug substance-polyelectrolyte interactions can affect drug performance through the alteration of transport and distribution processes [1-3]. Furthermore investigations aimed at utilizing glycosaminoglycans in drug delivery systems are carried out [4-7]. For polymer-based drug delivery vehicles thorough knowledge of drug-polyelectrolyte interactions is advantageous. Ionic or electrostatic interactions may constitute a key factor influencing the degree of drug incorporation [8, 9] and release rates from such delivery systems [4, 5, 10].

A range of methods have been applied to the study of low-molecular-weight ligand-polyelectrolyte complexation including equilibrium dialysis [11-13], isothermal titration calorimetry [14-16], ion selective electrodes [17, 18], and capillary electrophoresis (CE) [19-22]. CE has been established as an efficient micromethod for studying molecular interactions [23-28]. Different formats for conducting affinity CE have been developed; their pros and cons have been discussed previously [23, 26, 29-33]. The recognized advantages associated with the capillary electrophoresis frontal analysis (CE-FA) format (and affinity CE generally) are relatively short analysis times, simple method development, low sample consumption, and ease of automation. CE-FA has primarily been applied in studies of drug-albumin binding, e.g. [29, 34-43]. Also drug binding to α1-acid glycoprotein [35, 36], lipoproteins [44-46], liposomes [47, 48], and the polyelectrolyte dextran sulfate [19, 20] has been investigated by CE-FA.

Chondroitin sulfate is the most abundant glycosaminoglycan in cartilage and is widely distributed into body tissues and fluids [49]. It is a polysaccharide consisting of alternating N-acetyl galactosamine and glucuronic acids, respectively, with the N-acetyl galactosamine sulfated at the 4th or 6th position (Fig. 1). Chondroitin sulfate is known to interact with positively charged molecules [16, 50, 51]. In the search for novel intra-articular depot formulation principles for potential use in postoperative pain management and osteoarthritis [52], we are interested in chemical structures which can provide binding to the articular cartilage as a means for drug targeting and/or achievement of local sustained drug release. To this end, CE-FA was evaluated as a rapid approach for identifying and ranking low-molecular-weight ligands possessing affinity to the articular cartilage constituent chondroitin sulfate. Here we report on the development of a CE-FA method feasible for characterization of low-molecular-weight ligand-chondroitin sulfate binding. For the cationic model compounds lidocaine, propranolol, scopolamine, N-methyl scopolamine, N-butyl scopolamine, 2-propylisochinolinium, and methyl viologen (Fig. 1) binding isotherms were constructed and apparent association constants determined. It is noted that the model compounds were selected based on their availability in the lab and structural properties. The possible effect of glycosaminoglycan interactions on pharmacokinetic and pharmacological effects upon administration of these compounds was not considered.

MATERIALS AND METHODOLOGY

Chemicals

Chondroitin sulfate (sodium salt) from bovine cartilage, lidocaine hydrochloride monohydrate, N-butyl scopolamine

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bromide, N-methyl scopolamine bromide, and (±)propranolol hydrochloride were obtained from Sigma (St. Louis, MO, USA). Methyl viologen dichloride (paraquat dichloride, 1,1’-dimethyl-4, 4’-bipyridinium dichloride) and 2-propylisochinolinium bromide were obtained from Aldrich (Steinheim, Germany). Scopolamine hydrobromide trihydrate was purchased from Merck (Darmstadt, Germany). Purified water from a Milli-Q deionization unit (Millipore, Bedford, MA, USA) was used throughout.

**Apparatus**

Capillary electrophoresis was conducted using a Beckman PACE MDQ equipped with a diode array detector (Beckman, Fullerton, CA). UV detection was performed at 205, 206, or 209 nm depending on the UV absorbance maximum of the ligand. Electropherograms were recorded using the Beckman 24-Karat software. The uncoated fused silica (Polymicro Technologies, Phoenix, AZ, USA) and polyvinyl alcohol (PVA) coated capillaries (Agilent Technologies, Waldbronn, Germany) were 31.2 cm × 50 μm ID and with a length of 20 cm to the detector.

The pH measurements were performed using a Metrohm 744 pH Meter (Metrohm Ltd., Herisau, Switzerland).

**Buffer and Sample Preparation**

The buffer used for electrophoresis and samples was a 67 mM sodium phosphate buffer, pH 7.40. Samples contained 50.0 g/L chondroitin sulfate corresponding to a molar concentration of disaccharide units of 1.04 × 10⁻¹ M when the repeat unit of bovine cartilage chondroitin sulfate is assumed to have the molecular formula C₁₄H₂₀NNaO₁₄S. The sample concentrations of the low-molecular-weight ligands ranged from 10 to 500 μM. Standards covering the concentration range 2 or 5 to 500 μM were used to prepare calibration curves.

**CE-FA Experiments**

Uncoated fused silica capillaries were conditioned for 30 min each with 1 M sodium hydroxide, water, and electrophoresis buffer. The PVA coated capillary was conditioned for 10 min each with 10 mM phosphoric acid and the CE buffer. Between the measurements the uncoated capillaries were flushed for 1 min each with 0.1 M sodium hydroxide and phosphate buffer whereas the PVA coated capillary was rinsed with 10 mM phosphoric acid and phosphate buffer (pH 7.4) (1 min each). The sample tray and the capillary cartridge temperature were set to 25°C and

Fig. (1). Structures of the low-molecular-weight ligands lidocaine (I), propranolol (II), scopolamine (III), N-methyl scopolamine (IV), N-butyl scopolamine (V), 2-propylisochinolinium (VI), and methyl viologen (VII) and the glycosaminoglycan chondroitin-6-sulfate (VIII).
The apparent association constant, $K_{app}$, was determined from the slopes of the binding isotherms ($[L]_{free}$, the total ligand concentration $[L]_{total}$, and the molar concentration calculated from the measured free ligand concentration previously [19]. Briefly, the binding density, $v$, was calculated from the measured free ligand concentration $[L]_{free}$, the total ligand concentration $[L]_{total}$, and the molar concentration of chondroitin sulfate disaccharide units, $C_{CS}$:

$$v = \frac{[L]_{bound}}{C_{CS}} = \frac{([L]_{total} - [L]_{free})}{C_{CS}}$$ (1)

The apparent association constant, $K_{app}$, was determined from the slopes of the binding isotherms ($v$ vs $[L]_{free}$).

RESULTS AND DISCUSSION

Method Development

Initial experiments were conducted using propranolol as the ligand. Fig. (2) shows the effect of increasing injection times for propranolol standards and samples (propranolol and chondroitin sulfate) introduced by pressure (0.5 psi) from the short end of the capillary. Due to the different charge states the analyte is easily separated from the chondroitin sulfate. Frontal analysis conditions [53], where plateau peaks are observed, were achieved using injection times above 8 s and 5 s for standards and samples, respectively. In addition to propranolol, a UV signal for chondroitin sulfate and EOF was observed in the sample electropherograms (Fig. 2B). In CE-FA equilibrium is maintained during the separation due to the large sample volumes. The measured peak heights are proportional to the (free) ligand concentrations in the samples. Complexation between propranolol and the glycosaminoglycan is apparent as a decrease in plateau peak height and broadening of the propranolol peak in samples relative to the standards without chondroitin sulfate present (Fig. 2).

Qualitatively similar electropherograms were obtained for lidocaine, scopolamine, N-methyl scopolamine, N-butyl scopolamine, and 2-propylisochinolinium. In line with previous studies [19], strong adsorption to the fused silica capillary wall was observed for methyl viologen (parquat). Methyl viologen adsorbed onto PVA coated capillaries to a lesser extent indicating that the PVA coated capillary should be applied in the affinity studies encompassing methyl viologen. Upon selection of the operating conditions calibration curves were constructed. The between run repeatability of plateau peak heights was assessed and the R.S.D.s were below 3% for both standards and samples ($n = 3$) in most cases. A primary objective of the investigations was to develop a rapid method for assessing the complexation of low-molecular-weight ligands to chondroitin sulfate. Total analysis times (single standard/sample), including rinsing, and sample introduction were less than 5 min. Consequently, estimates of the degree of ligand complexation (requiring the analysis of one sample and one standard solution) can be obtained in 10 min.

![Fig. (2). Complexity of propranolol with chondroitin sulfate as studied by CE-FA. Electropherograms of 100 µM propranolol standards and samples containing 50 g/L chondroitin sulfate in 67 mM phosphate buffer (pH 7.40). (A) standards. (B) samples. Conditions: Uncoated fused silica capillary (31.2 cm × 50 µm ID, 11 cm effective length); applied voltage 8 kV (~47 µA); detection at 209 nm; hydrodynamic injection for 3-20 s (0.5 psi) from the short end; sample tray temperature 25°C; capillary cassette temperature 23°C.](image)

Ligand-Chondroitin Sulfate Complexation

The binding isotherms obtained for low-molecular-weight ligand-chondroitin sulfate complexation using CE-FA are depicted in Fig. (3). The isotherms depicting binding density $v$ as a function of the free ligand concentration were linear. In all cases $v$ was less than 0.01. It is known that for $v \rightarrow 0$, the binding equations reduce to $v/[L]_{free} = K_{app}$ [19, 54]. Thus, the apparent association constant $K_{app}$ was determined form the slopes of the binding isotherms. The determined $K_{app}$-values are summarized in Table I. Alternatively, the association constants may be determined by extrapolation ($v/[L]_{free} vs [L]_{free}$) as described by Klotz [54]. This approach was found to provide equivalent results (Table I).

The investigated ligands may be ranked with respect to their affinity for chondroitin sulfate according to the
Table 1. Degree of Complexation of Low-Molecular-Weight molecular-weight ligands with chondroitin sulfate (50 g/L) in 67 mM phosphate buffer (pH 7.40) at 25°C.

Table 1. Degree of Complexation of Low-Molecular-Weight ligands to Chondroitin Sulfate (50 g L⁻¹; 1.04 × 10⁻¹ M) and Apparent Association Constants, Kₐpp, Determined by Capillary Electrophoresis Frontal Analysis in 0.067 M Phosphate Buffer (pH 7.4) at 25°C

<table>
<thead>
<tr>
<th>Compound</th>
<th>Kₐpp (M⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lidocaine</td>
<td>13³/13⁶</td>
</tr>
<tr>
<td>Propranolol</td>
<td>23³/28⁸</td>
</tr>
<tr>
<td>Scopolamine</td>
<td>14⁴/14⁸</td>
</tr>
<tr>
<td>N-methyl scopolamine</td>
<td>13³/15⁶</td>
</tr>
<tr>
<td>N-butyl scopolamine</td>
<td>16¹/17⁷</td>
</tr>
<tr>
<td>2-Propylisocholininium</td>
<td>14¹/15⁵</td>
</tr>
<tr>
<td>Methyl viologen</td>
<td>28¹/29⁹</td>
</tr>
</tbody>
</table>

Kₐpp Determined from the slope of the binding isotherm.

*Kₐpp* Determined by extrapolation from plots of v/[L]ₜₜ as a function of [L]ₜₜ.

obtained Kₐpp-values: lidocaine = N-methyl scopolamine = scopolamine = 2-propylisocholininium < propranolol < methyl viologen. At the selected experimental conditions (pH 7.4 and 25°C), the low-molecular-weight ligands are positively charged and chondroitin sulfate is negatively charged (Fig. 1). The presence of chondroitin sulfate in the phosphate buffer was found to lower the pH to 7.26. Lidocaine, propranolol, and scopolamine have pKₐ values of 7.86, 9.45, and 7.55 [55], respectively, and the degree of ionization at pH 7.4 (7.26) is 0.74 (0.80), 0.99 (0.99), and 0.59 (0.66), respectively. N-methyl scopolamine and N-butyl scopolamine are permanently charged due to the presence of a quaternary amino group. 2-propylisocholininium and methyl viologen (paraquat) possess one and two permanently charged quaternary nitrogen functional groups, respectively. Except for propranolol, the ligands carrying only one charge/ionizable group displayed almost similar affinity for chondroitin sulfate. Electrostatic interactions are expected to play an important role in the complexation reaction [16, 50]. However, the lower degrees of ionization for lidocaine and scopolamine did not manifest in lower degrees of complexation (as compared to the other monovalent ligands). The fact that scopolamine and its quaternized derivatives exhibit similar affinity towards chondroitin sulfate indicates that sterical effect and/or hydrophobic effects are of minor importance or cancel out with the decreased fraction of ionized scopolamine. The cause for the relatively high degree of propranolol complexation is not clear but may be related to differences in lipophilicity or the presence of the β-hydroxy group which may participate in hydrogen bonding. As expected, and in agreement with previous studies on chondroitin sulfate binding comparing mono- and dicatonic species [50], methyl viologen exhibited the highest affinity for chondroitin sulfate. Santos et al. [16] also investigated the binding of propranolol to chondroitin sulfate using isothermal titration calorimetry, however, under different experimental conditions (ionic strength) making direct comparison difficult.

Ishwar et al. used mobility shift affinity CE to investigate the affinity of two dodecapeptides for chondroitin sulfate and heparin [53]. To the best of our knowledge this study represents the first application of CE-FA for investigation of chondroitin sulfate complexation. An advantage, at least in some applications, of CE-FA as compared mobility shift affinity CE may be that free ligand concentrations are provided directly as the output. CE-FA was found suitable for ranking and quantification of low-molecular-weight ligand complexation with chondroitin sulfate. With the experimental conditions applied in the current CE-FA study only the lower part of the chondroitin sulfate binding isotherm were characterized and, consequently, the results are not suitable for obtaining information on the number of ligand binding sites per glycosaminoglycan disaccharide unit in contrast to other methods reported [16, 50]. This might be accomplished for some ligands also by CE-FA through appropriate modification of the experimental conditions, i.e. adjustment of chondroitin sulfate concentration, ligand concentration and/or ionic strength. However, this was considered beyond the scope of the present study.

CONCLUSIONS

In the present study a CE-FA method feasible for studying low-molecular-weight ligand binding to the glycosaminoglycan chondroitin sulfate was developed. CE-FA is an attractive method for studying ligand-chondroitin sulfate interactions due to the low sample consumption and the short analysis times obtained. Ranking of low-molecular-weight ligand complexation with chondroitin sulfate was achieved based on CE-FA results. Except from propranolol the monovalent cationic ligands possessed almost similar affinity for chondroitin sulfate. The complexation of propranolol was intermediate between the single charged ligands and methyl viologen carrying two positive charges and exhibiting the strongest interaction with chondroitin sulfate. CE-FA may constitute a feasible tool for characterization of ligand-glycosaminoglycan complexation in drug research.

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**ABBREVIATIONS**

ACE = Affinity capillary electrophoresis  
CE = Capillary electrophoresis  
CE-FA = Capillary electrophoresis frontal analysis  
PVA = Polyvinyl alcohol

**REFERENCES**


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