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Analytical aspects of achiral and cyclodextrin-mediated capillary electrophoresis of warfarin and its two main derivatives assisted by theoretical modeling

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ABSTRACT

Several distinct analytical issues have been addressed by performing capillary electrophoresis-based separations of the warfarin, 7-hydroxywarfarin and 10-hydroxywarfarin in an achiral and cyclodextrin-containing media. The measurements were conducted across a range of pH in order to find optimum conditions for achiral and chiral separations. The values of acid dissociation constant (pK_a) have been determined and compared. Subsequently, after performing a series of mobility shift assays at different pH and cyclodextrin concentration, the pK_a values ascribed to diastereomeric complexes with methyl-β-cyclodextrin have been estimated. The significant pK_a shifts upon complexation have been noticed for warfarin – up to 1.5 pH units, and only subtle for 10-hydroxywarfarin. A new approach that allows the estimation of association percentage based on the electrophoretic mobility curves has been also demonstrated. The complex mechanism of chiral separation has been found to be responsible for the observed migration profile, relying on a combined equilibrium between complexation/partition and protonation/deprotonation phenomena. The occurrence of the pK_a-related migration order reversal has been demonstrated in achiral medium between warfarin and 7-hydroxywarfarin, and in chiral medium between enantiomers, causing a drop in enantioselectivity at specific pH. In parallel, the density functional theory-based calculations have been performed in order to obtain the structures of warfarin and its derivatives as well as to rationalize the shifts in pK_a values.

1. Introduction

The capillary electrophoresis (CE) is an instrumental separation technique characterized by high efficiency and general cost-effectiveness, which can be applied for multifarious bioanalytical purposes. Most commonly CE is used as a separation tool. In particular, it proves to be useful technique for the stereoselective separations [1–9]. Apart from an analysis of samples with constant composition, CE may be used to study the kinetic processes, e.g. to monitor the progress of diverse enzymatic reactions in time, or on the contrary, to measure the dynamics of non-covalent interactions in equilibrated mixtures [10–15]. Finally, it also allows one to determine the values of physicochemical parameters, e.g. an acid dissociation constant – pK_a, with fairly good accuracy [16].

The efficient separation of enantiomers by CE requires a difference in their effective electrophoretic mobility (μ_{eff}) values, which may result from dynamical interaction of analytes with chiral selector molecules. It has been proven that there are two independent mechanisms which may account for such effect: a different complexation percentage caused by unequal affinity of enantiomers, and a different μ_{eff} of diastereomeric complexes related to unequal ionization level and/or a different molecular shape [17]. The combined complexation/partition and protonation/deprotonation equilibria correspond to the four distinct subspecies of analyte which may exist, and the final enantioresolution effect is defined by the respective probabilities of analyte to be present in particular form. Therefore, description of chiral separation mechanism based only on the complexation equilibrium may be incomplete, especially when after complexation the enantioselective pK_a shifts are observed. The complexation-related selectivity and ionization-related selectivity may cooperate or counteract each other. In some cases, at specific pH or chiral selector concentration values, the enantiomeric migration order reversal (MOR) may occur.

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The theory of combined equilibrium and possible pK_a shifts upon complexation have been proposed two decades ago, and further developed by Rizzi's and Vigh's groups [18–25]. Besides the thorough theoretical description, the pK_a shift-related effects have been used in a chiral isoelectric focusing method, where zwitterionic diastereomeric complexes differ in p*I* values enabling their separation [18,24]. Interestingly, in spite of the well elucidated theory there are relatively a few reported examples on the combined equilibrium mechanism, enantioselective pK_a shifts, and other related phenomena accounting for enantiomers separation by CE [18–32].

Warfarin (WAR) [3-(α-acetonylbenzyl)-4-hydroxycoumarin] is a widely used anticoagulant drug undergoing multilateral metabolism driven by several different isoforms of cytochrome P450 [33]. It makes the group of WAR oxidative metabolites very large, and structurally diverse. Hydroxywarfarins (WAR-OHs) posses the second hydroxyl group bounded to different sites of WAR molecules, and consequently, may exert the significant differences in physicochemical properties.

Separation of the WAR enantiomers by CE was the frequently addressed problem, solved by several distinct approaches, mainly by addition of the cyclodextrins (CDs) to background electrolyte (BGE) as chiral selectors [34–44]. The pK_a of WAR was determined several times by CE, including the recent attempts to estimate pK_a shifts resulting from the supramolecular interaction with β-CD and albumin [45–50]. WAR-OHs, contrary to the parent compound, may display double protonation/deprotonation equilibrium in solution. To the best of our knowledge, the pK_a values characterizing WAR-OHs have not yet been determined experimentally.

The novelty of our studies presented herein comprises: (*i*) investigation of the selectivity and enantioselectivity variance across a range of pH, and thus, finding optimal pH values for achiral and chiral separations of WAR and its two derivatives; (*ii*) determination of their pK_a values; (*iii*) determination of total pK_a shifts characterizing the formation of diastereomeric inclusion complexes with methyl-β-cyclodextrin (Me-β-CD); (*iv*) construction of the association affinity model providing the insight into the fraction of associated molecules during separation based only on the μ_{eff} values; (*v*) evaluation of the chiral separation mechanism in the context of the two independent stereoselective phenomena, i.e. association and ionization; (*vi*) and finally, in silico calculations based on the density functional theory to enable a deeper insight into a possible structure–property relationship.

2. Materials and methods

WAR (racemic mixture) was supplied by Sigma–Aldrich (Germany), 7-hydroxywarfarin (WAR-7-OH) and 10-hydroxywarfarin (WAR-10-OH, both racemic mixtures) by LGC Standards (USA), while all other chemicals except CDs by Avantor Performance Materials Poland. S.A. (Poland). 2-Hydroxypropyl-β-cyclodextrin (2-HP-β-CD, 0.5–1.3 substituted groups per glucose unit) and Me-β-CD, 1.7–2.1 substituted groups per glucose unit), were supplied by Sigma–Aldrich (Germany).

The CE experiments were carried out using normal polarity on the P/ACE MDQ Capillary Electrophoresis System (Beckman Coulter, USA) equipped with a diode array detector. All solutions were prepared in the deionized water (MilliQ, Merck-Millipore) and filtered through 0.45 μm regenerated cellulose membrane, then degassed by centrifugation. Sample injection was conducted using a forward pressure at anodic side applying: 0.5 psi for 5 s, unless stated otherwise. The standard solution concentrations were 0.100 mg/mL for WAR, 0.075 mg/mL for WAR-10-OH and 0.050 mg/mL for WAR-7-OH (aqueous solutions). Dimethyl sulfoxide (DMSO) was used as the EOF marker, and was added to samples in final concentration 0.2% (v/v). During all experiments the whole spectra within the range of 200–600 nm were collected, the results obtained at 210 or 310 nm were used to the further processing. An uncoated fused-silica capillary was a 60 cm × 50 μm i.d. one (Beckman Coulter, USA), with a 50 cm distance to the detector. The sample tray and the capillary were thermostated at 22 °C using the sample garage and the liquid cooling system, respectively.

The rinsing procedure between runs included: 20 psi (0.138 MPa) of 10 mM lauryldimethylamine-oxide (LDAO, aqueous solution) for 2 min, 20 psi of deionized water for 1 min, 20 psi of 0.1 M NaOH for 2 min, and 20 psi of BGE for 2 min; while during the first use of the capillary at a working day: 20 psi of methanol for 5 min, 20 psi of 10 mM LDAO for 5 min, 20 psi of deionized water for 2 min, 20 psi of 0.1 M HCl for 2 min, 20 psi of 0.1 M NaOH for 10 min, and 20 psi of BGE for 10 min. For the fresh capillary conditioning, the latter sequence was used, but the duration of each individual step was doubled.

The buffers were prepared according to the instructions received from the PHoEBuS 1.3 software by Analis (Belgium), applied in order to obtain the same ionic strength of all buffer solutions (50 mM). Then, their actual pH values were measured. The change of viscosity with regard to the increasing pH of buffers has been evaluated by performing additional experiment, but it has occurred to be of no significance. All essential information for the buffer preparation can be found in Table S-1 in the Supplementary Material. When performing separations in the chiral media, the chosen CD was used as the buffer additive, in standard 1% (w/v) concentration, except the experiment assuming different Me-β-CD concentrations (see the Supplementary Material for more details). All quantitative outcomes have been calculated as an average from at least three repetitions within the same experimental conditions.

3. Results and discussion

3.1. Achiral separations

The separations of WAR, WAR-7-OH and WAR-10-OH have been performed in an extensive series of buffers at different pH. The μ_{eff} values have been calculated from the migration times, using DMSO as a chosen electroosmotic flow (EOF) marker, according to Eq. (1).

$$\mu_{eff} = \mu_{obs} - \mu_{eof} = \frac{L_{tot} \cdot L_{eff}}{V} \cdot \left(\frac{1}{t_{obs}} - \frac{1}{t_{eof}} \right) \quad (1)$$

where μ_{eff} and μ_{obs}, are the effective and observed or apparent electrophoretic mobilities of analyte ($cm^2\ V^{-1}\ s^{-1}$), respectively; μ_{eof} is the mobility of electroosmotic flow ($cm^2\ V^{-1}\ s^{-1}$); L_{tot} and L_{eff} are the total and effective capillary lengths (cm), respectively, V is the separation voltage (V); t_{obs} is the measured migration time of analyte (s), while t_{eof} is the time measured for neutral marker–DMSO (s).

This experiment enabled us to measure the selectivity coefficient value α, defined as the ratio of the difference of the effective mobilities of two analytes divided by the mobility of one, as the function of pH, which has been presented in Fig. 1A. The results indicate that the highest selectivity is obtained between WAR-10-OH and WAR, although, the efficient separation between WAR and WAR-10-OH is hampered starting from pH 8.0. At pH around 8.0 WAR and WAR-7-OH are virtually indistinguishable, since their migration order changes near this pH value – the selectivity coefficient changes its sign. Previously, this behavior was unknown. The optimal conditions for separation of WAR, WAR-7-OH and WAR-10-OH mixed together have been found at pH range 4.0–6.0, where all the selectivity coefficient values are relatively high (electropherogram presented in Fig. 2A). The results obtained in this part enable fast selection of pH for an achiral method optimization regarding selectivity as a decisive parameter.