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Determination of acid dissociation constants of warfarin and hydroxywarfarins by capillary electrophoresis

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ABSTRACT

In this work the acid dissociation constants – pK_a of warfarin and its all important oxidative metabolites have been determined by capillary electrophoresis-based methods. It has resulted in a complete description of two acid–base dissociation equilibria, yet not investigated experimentally for phase I metabolites of warfarin. The capillary electrophoresis (CE) method based on the relation between effective electrophoretic mobilities and pH has proven to be a suitable tool for pK_a determination, while the spectrophotometric (CE-DAD) and the internal standard methods (IS-CE), have appeared to be promising alternative approaches. The CE-DAD approach based on the change in absorbance spectra between the acidic and basic forms is a combination between capillary electrophoresis and spectrophotometric titration, and yields very consistent values of pK_{a1} with CE. The IS-CE, in turn, enables an estimation of pK_{a1} and pK_{a2} from only two analytical runs, however, less accurate than CE and CE-DAD. The Debye–Hückel model has been confirmed experimentally as a good predictor of pK_a values at various ionic strengths. Therefore, it has been used in determination of thermodynamic pK_{a1} and pK_{a2}, referring to the zero ionic strength. The results are important from the analytical, pharmacological, and theoretical points of view.

1. Introduction

Warfarin (WAR) [3-(α-acetonylbenzyl)-4-hydroxycoumarin] is a widely used drug exhibiting anticoagulant activity, applied in the prevention of thrombosis and thromboembolism, i.e. formation of blood clots and vascular occlusions [1,2]. Its routine application as an effective anticoagulant is however quite problematic owing to complex oxidative metabolism, involving several different isoforms of cytochrome P450 enzyme (CYP). The main oxidative metabolites of WAR are hydroxywarfarins: 3′-hydroxywarfarin (W3), 4′-hydroxywarfarin (W4), 6-hydroxywarfarin (W6), 7-hydroxywarfarin (W7), 8-hydroxywarfarin (W8) and 10-hydroxywarfarin (W10). CYP-mediated metabolism of WAR is highly regio- and enantioselective, the most popular hydroxywarfarins found *in vivo* are (*S*)-W7, (*R*)-W4 and (*R*)-W10 [1].

The acid dissociation constant, usually expressed as its pK_a value, is one of the most important parameters describing physicochemical properties of drugs [3,4]. It characterizes acid–base equilibrium of the respective ionizable groups in solution, thus, indicates their dissociation (deprotonation) potential at a given pH. The acidic and basic forms of drugs differ in charge – at least one of them is ionized, therefore, they also may differ in other key properties like water solubility, membrane permeability, affinity of association with proteins, and finally, therapeutic activities. As it was demonstrated in the literature, therapeutic activity of WAR is also dependent on its ionization level [2]. For that reason, the exact value of pK_a for WAR is important from the pharmacological point of view, and it was determined many times in the past [5–9]. Another issue is acid–base properties of WAR metabolites, yet not fully recognized. Hydroxywarfarins, contrary to the parent drug, are supposed to be capable of a double dissociation, and this fact complicates predictions of their ionization-related properties. Therefore, experimental determination of their exact pK_{a1} and pK_{a2} values is undeniably necessary for the holistic understanding of WAR pharmacokinetics.

There are many various analytical techniques that allow for accurate estimation of pK_a values in a sound and rapid way [3,10]. Capillary electrophoresis (CE) belongs to the subgroup of separation

Abbreviations: DMSO, dimethyl sulfoxide; EOF, electroosmotic flow; WAR, warfarin; W3, 3′-hydroxywarfarin; W4, 4′-hydroxywarfarin; W6, 6-hydroxywarfarin; W7, 7-hydroxywarfarin; W8, 8-hydroxywarfarin; W10, 10-hydroxywarfarin.

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techniques, and in the recent years, has gained particular interest as an efficient tool for pK_a determination [11–17]. Its growing popularity originates from the clear analytical benefits: (i) very small consumption of samples and buffers; (ii) relatively high throughput and simple automation; (iii) possibility to separate the sample component from each other, including impurities; (iv) and the accuracy, typically within 0.05 pH units. In a standard approach based on CE, pK_a values are calculated from a curve presenting dependence of electrophoretic mobility on pH. For comparative purposes, the use of thermodynamic pK_a (p$K_a{}^T$) corresponding to the zero ionic strength and standard temperature 25 °C is more suitable than the apparent pK_a, determined experimentally in specific conditions of ionic strength and temperature [18,19].

In this work, the acid–base equilibria of WAR and six hydroxywarfarins have been investigated and characterized by determination of the respective pK_a values. To the best of our knowledge, this is the first experimental study of all pharmacologically important hydroxywarfarins, and in addition, it covers both dissociation equilibria. The standard CE-based method has been used to obtain the most accurate results. Afterwards, usefulness of two alternative approaches utilizing CE have been investigated: the spectrophotometric method (CE-DAD), based on the changes in molecular spectra recorded by DAD detection system; and the internal standard-based method (IS-CE), restricted only to two analytical runs and the known pK_a value of an internal standard. The variation of pK_{a1} with changing ionic strength has been studied experimentally, and compared with the values predicted by the Debye–Hückel model. Finally, p$K_{a1}{}^T$ and p$K_{a2}{}^T$ values have been found for each compound, which correspond to the standardized temperature and zero ionic strength. It enables creation of a more complete picture of pharmacologically-important properties of WAR metabolites, theoretical considerations referring to structure–property relationships, and better understanding of their migration profile observed during CE separations.

2. Material and methods

2.1. Instrumentation

WAR (racemic mixture) was supplied by Sigma–Aldrich (St. Louis, MO, USA), W3, W4, W6, W7, W8, and W10 (all racemic mixtures) by LGC Standards (Teddington, UK), while all other chemicals by Avantor Performance Materials Poland S.A. (Gliwice, Poland). All standard solutions were prepared in the deionized water (MilliQ, Merck-Millipore Billerica, MA, USA) and filtered through the 0.45 μm regenerated cellulose membrane, then degassed by centrifugation. Standard concentration of analytes was 0.1 mg/mL, all analytes were dissolved in water/methanol (1:1 v/v) mixture. Dimethyl sulfoxide (DMSO) was used as the electroosmotic flow (EOF) marker, in 0.2% (v/v) concentration.

The P/ACE MDQ Capillary Electrophoresis System (Brea, CA, USA) equipped with a diode array detector was used for all CE-based experiments, using the bare fused-silica capillary of 60 cm total length and 75 μm internal diameter. Separations were conducted in a short-injection mode [20], unless stated otherwise, using a 10 cm long outlet capillary part. Owing to this fact, the total separation time could be vastly reduced. Sample injection was conducted using forward pressure 0.4 psi for 4 s. During separations, 15 kV voltage (anode at injection end) and the additional forward pressure of 0.4 psi were applied. The current measured during separations was between 25 and 125 μA. Capillary was conditioned at 25 °C, using the liquid cooling system. Every time, DAD detector collected the whole spectra within 200–600 nm. Signal recorded at 200, 280 and 308 nm was used for the further analysis.

Table 1
Composition and predicted pH of all buffering solutions prepared for experiments, calculated for 50 mL total volume and 100 mM ionic strength.

pH	Buffer composition (mL)	
Phosphate buffer I	H_3PO_4 (100 mM)	NaH_2PO_4 (100 mM)
2.50	18.52	4.80
Acetic buffer	CH_3COOH (500 mM)	CH_3COONa (500 mM)
4.50	14.16	10.00
5.25	2.52	10.00
Phosphate buffer II	NaH_2PO_4 (100 mM)	Na_2HPO_4 (100 mM)
6.00	3.59	4.70
7.00	1.01	13.29
8.00	0.12	16.25
Borate buffer	$Na_2B_4O_7{\cdot}10H_2O$ (50 mM)	NaOH (1 M)
9.40	39.77	1.02
10.00	28.67	2.13
11.00	25.05	2.50
NaOH/KCl	NaOH (1 M)	KCl (1 M)
12.00	0.64	4.36
13.00	4.55	0.45

Capillary rinsing was conducted between runs applying pressure of 137.9 kPa (20 psi). The particular steps of rinsing were: deionized water for 1 min, 0.1 M NaOH for 2 min, and background electrolyte (BGE) for 2 min. During the first use of the capillary at a working day: methanol for 5 min, 0.1 M HCl for 2 min, deionized water for 2 min, 0.1 M NaOH for 10 min, and BGE for 10 min were applied. For the fresh capillary conditioning, the latter sequence was used, but the duration of each individual step was doubled.

2.2. Buffering solutions

Composition of the given BGEs was calculated with the use of PHoEBuS 1.3 software by Analis (Namur, Belgium), setting 100 mM as the selected value of ionic strength. The buffers of lower ionic strength were prepared by dilution with deionized water. The obtained recipes for BGEs preparation have been presented in Table 1. The values of pH were verified experimentally prior to CE separations.

2.3. Methods for pK_a determination

2.3.1. Standard electrophoretic method (CE)

In the standard CE approach, pK_a values have been estimated by applying the non-linear regression model to the obtained dependency between effective electrophoretic mobility (μ_{eff}) and pH. According to the Eq. (1), μ_{eff} can be calculated from the respective migrations times of analyte and EOF marker:

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

where μ_{eff} and μ_{obs} are the effective and observed electrophoretic mobilities of analyte ($m^2\,V^{-1}\,s^{-1}$), respectively; μ_{eof} is the mobility of electroosmotic flow ($m^2\,V^{-1}\,s^{-1}$); L_{tot} and L_{eff} are the total and effective capillary lengths (m), 0.60 and 0.10 m, 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 of EOF – DMSO (s).

The relation between μ_{eff} and pH is described by Eqs. (2) and (3), for the monoprotic and diprotic acid, respectively:

$$\mu_{eff} = \left[\frac{\alpha \cdot 10^{-pK_a}}{10^{-pK_a} + 10^{-pH}} \right] \quad (2)$$

$$\mu_{eff} = \left[\frac{\alpha_1 \cdot 10^{-pK_{a1}} \cdot 10^{-pH} + \alpha_2 \cdot 10^{-pK_{a1}} \cdot 10^{-pK_{a2}}}{(10^{-pH})^2 + 10^{-pK_{a1}} \cdot 10^{-pH} + 10^{-pK_{a1}} \cdot 10^{-pK_{a2}}} \right] \quad (3)$$

where α values are fitting constants equal to electrophoretic mobility of the deprotonated form of the acid with subscript 1 and 2 equal