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Investigation of the acid-base and electromigration properties of 5-azacytosine derivatives using capillary electrophoresis and density functional theory calculations

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ABSTRACT

Capillary electrophoresis (CE) and quantum mechanical density functional theory (DFT) were applied to the investigation of the acid-base and electromigration properties of important compounds: newly synthesized derivatives of 5-azacytosine – analogs of efficient antiviral drug cidofovir. These compounds exhibit a strong antiviral activity and they are considered as potential new antiviral agents. For their characterization and application, it is necessary to know their acid-base properties, particularly the acidity constants (pK_a) of their ionogenic groups (the basic N^3 atom of the triazine ring and the acidic phosphonic acid group in the alkyl chain). First, the mixed acidity constants (pK_a^{mix}) of these ionogenic groups and the ionic mobilities of these compounds were determined by nonlinear regression analysis of the pH dependence of their effective electrophoretic mobilities. Effective mobilities were measured by CE in a series of background electrolytes in a wide pH range (2.0-10.5), at constant ionic strength (25 mM) and constant temperature (25 °C). Subsequently, the pK_a^{mix} values were recalculated to thermodynamic pK_a values using the Debye-Hückel theory. The thermodynamic pK_a value of the NH^+ moiety at the N^3 atom of the triazine ring was found to be in the range 2.82–3.30, whereas the pK_a of the hydrogenphosphonate group reached values from 7.19 to 7.47, depending on the structure of the analyzed compounds. These experimentally determined pK_a values were in good agreement with those calculated by quantum mechanical DFT. In addition, DFT calculations revealed that from the four nitrogen atoms in the 5-azacytosine moiety, the N^3 atom of the triazine ring is preferentially protonated. Effective charges of analyzed compounds ranged from zero or close-to-zero values at pH 2 to −2 elementary charges at pH ≥ 9. Ionic mobilities were in the range $(-16.7 \text{ to } -19.1) \times 10^{-9}\ m^2\ V^{-1}\ s^{-1}$ for univalent anions and in the interval $(-26.9 \text{ to } -30.3) \times 10^{-9}\ m^2\ V^{-1}\ s^{-1}$ for divalent anions.

1. Introduction

The newly synthesized 5-azacytosine derivatives investigated in this work are important compounds belonging to the analogs of cidofovir – an efficient antiviral drug. Cidofovir, 1-(*S*)-[3-hydroxy-2-(phosphonomethoxy)propyl]cytosine is a potent antiviral agent, which is active against all types of DNA viruses [1]. As the systemic drug Vistide™, it has been approved in clinical practice for the treatment of cytomegalovirus retinitis in acquired immunodeficiency syndrome (AIDS) patients. It can also be clinically used off-label to treat severe cases of (malignizing) papillomatoses, progressive multifocal leukoencephalopathy, adenovirus and herpes virus infections as well as some rather obscure severe infections caused by poxviruses (vaccinia, orf, and molluscum contagiosum). The attractiveness of cidofovir is dramatically enhanced by its supreme activity against the smallpox virus and the related monkeypox virus [2].

However, the potential nephrotoxicity and poor bioavailability of cidofovir limit its widespread use [3]. The unique activity of cidofovir combined with the effort for diminution of its side effects led to subsequent development of cidofovir derivatives.

Abbreviations: BGE, background electrolyte; DFT, density functional theory; DMSO, dimethylsulfoxide; EOF, electroosmotic flow; MAD, median absolute deviation.

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The substitutions of various aliphatic components in the positions C-5 and N^4 result in the complete loss of antiviral activity [4,5]. However, the introduction of an additional nitrogen atom into the heterocyclic ring proved to be useful – 5-azacytosine derivatives exhibited similar or in some cases even higher antiviral activity than cidofovir. The antiviral selectivity index (the ratio of 50% cytotoxic concentration (CC_{50}) to 50% effective concentration (EC_{50})) of 5-azacytosine analog of cidofovir, 1-(*S*)-[3-hydroxy-2-(phosphonomethoxy)propyl]-5-azacytosine (**1**), was 2- to 16-fold higher than that of cidofovir [6]. This activity was further increased by the transformation of **1** to appropriate ester prodrugs [7].

The recent preparation of new C-6- and N^4-derivatives of 5-azacytosine [8–10] and their development into a potential medicament necessitates the knowledge of the physicochemical characteristics of these compounds, such as water solubility, lipophilicity and acid-base properties. The acid-base properties are quantitatively characterized by the acid dissociation constant or acidity constant, pK_a. The acidity constant is the key parameter for the comprehension of drug passage across the cell membrane, the estimation of ionic forms concentrations, the investigation of biological uptake and metabolism mechanism. Moreover, pK_a explains reactivity, the reaction rate and salt creation. In addition, the knowledge of the pK_a of compounds is also important for the selection of suitable experimental conditions during the development of new electromigration and chromatographic methods for their analysis and characterization [11,12].

The determination of the pK_a requires exposition of the compound to an environment of changing pH and the monitoring of a particular property that changes as a function of the ionization state of the molecule. There are several methods for determination of acidity constants [13–15]. Traditionally, potentiometric and spectrophotometric titrations were used for the determination of the acid dissociation constants of ionogenic compounds. The drawback of these classical methods is that they need a relatively large amount of a compound in a high purity degree. Moreover, spectrophotometric techniques require a compound to possess a UV or Vis chromophore with pH-dependent absorption. In addition to other analytical methods, e.g. NMR spectroscopy, mass spectrometry, calorimetry, surface plasmon resonance [13–15] and theoretical calculations [16,17], also electromigration methods – capillary isotachophoresis [18,19] and capillary electrophoresis (CE) are applied to pK_a determination [20–22]. As compared to the above potentiometric and spectrophotometric titration methods, CE provides several advantages: extremely low amounts of investigated compounds (microliter volumes at submillimolar to millimolar concentration) are sufficient; small volumes of aqueous buffers are used as background electrolytes (BGEs); the analysis of low soluble compounds is possible; the analytes need not be quite pure if their admixtures can be separated during the CE run; the precise knowledge of analyte concentration is not necessary, several analytes can be studied simultaneously; the method is relatively simple, can be automated, and is cost-effective and environment-friendly. The problem of long analysis times due to the suppressed electroosmotic flow (EOF) in CE analyses in strongly acidic BGEs is solved by the acceleration of the EOF mobility measurement by pressure [23] or vacuum [24], or by injection from the shorter capillary end (the reduction of the effective length) [25,26]. A higher throughput for the CE determination of the pK_a was achieved by employing weak acids and bases as the internal standards of the pK_a values [27–30]. The pK_a values were mostly determined by CE in aqueous media but CE estimation of pK_a in nonaqueous [31,32] and hydro-organic solvents [32,33] is possible as well.

Recent applications include the determination of thermodynamic or apparent pK_a values of variable compounds, e.g. amino- and guanidinopurine nucleotide analogs [34], benzimidazole and its derivatives [35]; very weak zwitterionic heterocyclic bases [36], 3-nitro-tyrosine and 3-chloro-tyrosine [37], tetrabromophenolphthalein ethyl ester [38], polyprotic peptide hormones [39], triazole fungicides [23] and different types of pharmaceuticals [11,27–29,40–44].

The aim of this work was to study the acid-base and electromigration properties of selected 5-azacytosine derivatives (see Fig. 1 and Table 1); particularly, to employ the experimental CE method and theoretical quantum mechanical density functional theory (DFT) calculation for the determination of the thermodynamic pK_a of their ionogenic groups and in this way to investigate the influence of the structure of these compounds, especially the presence of various substituents on C-6 and N^4 atoms, on their acid-base properties. In addition, from the CE measurements of the pH dependence of effective electrophoretic mobilities, the electromigration properties of analyzed compounds, effective charges and ionic mobilities, should be estimated.

2. Materials and methods

2.1. Chemicals

All the chemicals used were of analytical reagent grade. Sodium hydroxide, formic acid, acetic acid (AcOH), phosphoric acid and dimethylsulfoxide (DMSO) were supplied by Lachema (Brno, Czech Republic). CAPS (3-[cyclohexylamino]-1-propanesulfonic acid), CHES (2-[N-cyclohexylamino]ethanesulfonic acid), MES (2-morpholinoethanesulfonic acid) and MOPS (4-morpholinopropanesulfonic acid) were obtained from Serva (Heidelberg, Germany). Tricine (N-[tris(hydroxymethyl)]methylglycine) and Tris (tris[hydroxymethyl]aminomethane) were provided by Merck (Darmstadt, Germany).

2.2. Analyzed compounds

The analyzed 5-azacytosine derivatives were synthesized at our Institute following the procedures described elsewhere [8–10]. The synthetic products were purified by preparative HPLC and characterized by MS and NMR. The names, identification numbers and relative molecular masses (M_r) of analyzed 5-azacytosine derivatives are presented in Table 1 and their structures are shown in Fig. 1. Compound **1** is the basic structure, compounds **2**–**5** contain an aliphatic alkyl substituent (methyl, ethyl, propyl and butyl) on the C-6 atom, compound **6** possesses an aromatic phenyl substituent on the C-6 atom, and compound **7** differs by cyclopropyl group on the N^4 atom. For the atom numbering of the analyzed compounds see Fig. 2 below.

2.3. Capillary electrophoresis

CE analyses were carried out using the P/ACE MDQ Capillary Electrophoresis System (Beckman Coulter, Fullerton, CA, USA) and the 32 Karat Software (Beckman Coulter, Fullerton, CA, USA) for the system control and data acquisition and processing. Separations were performed in an internally untreated fused-silica capillary with outer polyimide coating (Polymicro Technologies, Phoenix, AR, USA), with I.D./O.D. 50/375 μm and the total/effective length 395/294 mm. The analytes were detected by a UV–vis photodiode array detector operating in the range 190–300 nm. The absorbance of the analyzed compounds and DMSO (used as an EOF marker) was monitored at 206 and 254 nm. The analyses were performed at a constant temperature of BGE inside the capillary (25 °C) by an active cooling of the capillary.

The effective mobilities of 5-azacytosine derivatives were measured in a broad pH range (2.00–10.50) of aqueous BGEs at a constant ionic strength 25 mM. The pH of the buffer solutions was