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Received November 4, 2011
Revised March 2, 2012
Accepted March 2, 2012

Research Article

Study of DNA–ellipticine interaction by capillary electrophoresis with laser-induced fluorescence detection

Ellipticine (5,11-dimethyl-6H-pyrido[4,3-b]carbazole), an alkaloid isolated from Apocynaceae plants, exhibits an antitumor activity, which is exceptionally high against several specific types of tumors. Ellipticine is also interesting as an anticancer drug as it has limited side effects and lacks of hematological toxicity. Various methods to study intercalating activity of this drug have been developed. However, to our best knowledge, capillary electrophoresis (CE) as a technique combining high separation resolution with various detection options has never been used for these purposes. In this study, a novel separation method based on CE with laser-induced fluorescence (CE-LIF) detection has been developed for the determination of ellipticine and for the monitoring of ellipticine–DNA interaction. Sodium acetate (50 mM, pH 4.5) was used as a background electrolyte and LIF detection at $\lambda_{ex} = 488$ nm. The limit of detection for ellipticine was determined to be 5×10^{-8} M. A total of 20% dimethyl sulfoxide was found optimal as sample solvent. Additionally, intercalation of ellipticine into the double-stranded DNA was investigated. Signal corresponding to ellipticine was decreasing and a new peak appeared and was growing. It can be concluded that CE-LIF is a method applicable to in vitro studies of ellipticine–DNA complexes.

Keywords:
Capillary electrophoresis / DNA / Drugs / Ellipticine / Tumor disease
DOI 10.1002/elps.201200056

1 Introduction

Ellipticine (5,11-dimethyl-6H-pyrido[4,3-b]carbazole), an alkaloid isolated from Apocynaceae plants, exhibits significant antitumor and anti-HIV activities [1–3]. High efficiencies against several types of cancer of ellipticine and their derivatives, their limited toxic side effects, and their lack of hematological toxicity belong to the main reasons for the interest in these compounds in treatment [4]. The ellipticine and its derivatives act as potent antitumor agents via a combined mechanism of cell cycle arrest and induction of the apoptotic pathway [5–7]. Ellipticine might also act as a modulator of p53 nuclear localization as it was shown in HCT116 colon cancer cells, in which ellipticine increased the nuclear localization of endogenous p53 causing the transactivation of the p21 promoter [8]. Recently, it has been found that ellipticine also activates the p53 pathway in glioblastoma cells, in which its impact on these cancer cells depends on the p53 status. In a U87MG glioblastoma cell line expressing p53wt, ellipticine provoked an early G0/G1 cell cycle arrest, whereas in a U373 cell line expressing p53mt, it caused arrest in S and G2/M phases [9]. Nevertheless, the precise molecular mechanism responsible for these effects has not been explained yet.

It is not surprising that numerous methods including surface-enhanced Raman spectroscopy [10], electrochemical techniques [11–13], and spectrophotometric methods [14,15] have been used for both determination of ellipticine and its derivatives and studying of their interactions with some biomolecules, mainly with DNA. Moreover, several biological methods have been used to investigate a mechanism of ellipticine-mediated DNA damage such as intercalation into DNA and/or formation of covalent DNA adducts and have been reviewed in [4,16,17]. Covalent binding of ellipticine after its enzymatic activation and quantitation of adducted nucleotides per normal nucleotide is specifically determined by the ^{32}P-postlabelling technique [18–25], methods utilized for the analysis of its intercalating activity need to be further investigated because of direct quantification of ellipticine bound into DNA. Recently, adsorptive transfer stripping technique coupled with square wave voltammetry to study the interaction of ellipticine with dsDNA was utilized [26]. This method has been found to be suitable to detect and quantify ellipticine intercalation into dsDNA. There have been also suggested methods as microspectrofluorimetry [27] and time-resolved

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fluorescence spectroscopy [28], which utilize the fact that ellipticine is fluorescent dye. Detailed studies of ellipticine fluorescence has been carried out under various conditions such as pH and solvent composition [29–33] as well as in the presence of selected anions including fluoride and acetate [32]. There have also been published some attempts to employ liquid chromatography [34–37] and gel electrophoresis [38] for ellipticine analysis, but, to our knowledge, CE has never been utilized for this purpose. Among electrophoretic methods, CE with laser-induced fluorescence (CE-LIF) belongs to the most sensitive one. Moreover, CE-LIF can be miniaturized and the manufactured instrument is potentially applicable as a point-of-care method. Therefore, the investigation of the potential of CE-LIF for detection of ellipticine was the general aim of this work. Moreover, we optimized and demonstrated for the first time a novel method based on CE-LIF detection for the studying of ellipticine intercalation.

2 Materials and methods

2.1 Chemicals

Ellipticine, DMSO, acetic acid, sodium hydroxide (NaOH), and other chemicals were purchased from Sigma Aldrich (St. Louis, MO, USA) in ACS quality unless noted otherwise. The solution of ellipticine was prepared by 10-fold dilution of the stock solution (33.7 mM in DMSO) with sodium acetate (10 mM, pH 4.5). This solution was used for further dilution with DMSO or water prior to analysis. Lyophilized highly polymerized DNA (Reanal, Hungary) was isolated from chicken erythrocytes (Mw = 400 000 g/mol). The stock solution of DNA (1 mg/mL) was prepared by dissolving in ACS water. All other solutions, such as acetate buffers (10, 50, and 100 mM, pH 4.5), were prepared in MiliQ water. Deionized water underwent demineralization by reverse osmosis using the instrument Aqua Osmotic 02 (Aqua Osmotic, Tisnov, Czech Republic) and then subsequently purified using Millipore RG (Merck Millipore, Billerica, MA, USA, 18 M′Ω)—MilliQ water. The pH values were measured using pH-meter inoLab Level 3 (Wissenschaftlich-Technische Werkstatten GmbH; Weilheim, Germany).

2.2 Capillary electrophoresis

All analyses were carried out using CE instrument (P/ACE 5500, Beckman Coulter, Breaca, CA, USA) equipped with LIF detector using argon ion laser with excitation wavelength of 488 nm. The emission above 510 nm was registered. Separations were performed in an uncoated fused silica capillary (Polymicro Technologies, Phoenix, AZ, USA) with an internal diameter of 75 μm and external diameter of 375 μm. The total length of the capillary was 46 cm and the effective length was 39 cm. The capillary was flushed with 0.1 M NaOH for 5 min and with BGE for 10 min prior to the first use. The capillary was rinsed for 2 min with BGE before each run to obtain stable and repeatable measurements. Sample was injected hydrodynamically by the pressure of 3.4 kPa applied for 20 s. Separation voltage was set to 20 kV. Sodium acetate buffer (pH 4.5) was used as a BGE. To monitor the interaction between dsDNA and ellipticine, a solution of DNA (1 mg/mL in water) was mixed with the ellipticine solution (30 μM in 100% DMSO) in 1:1 ratio (v:v). This mixture was subsequently measured in 10-min intervals.

3 Results and discussion

3.1 CE method development and optimization

Ellipticine dissolved in water exists in four forms: one zwitterionic, one neutral, and two ionic forms (Fig. 1). Both of the nitrogen atoms are responsible for presence of charged forms of ellipticine, which is convenient for charged-based separation methods. The molecule is pH sensitive with two protonable ring nitrogen atoms at positions 2 and 6. The p*K*a of N-6 is less than 1, and that of N-2 is about 6, although it can vary from 4.5 to 7.4 depending on the nature and position of substituents on the ring system. Generally, both protonated and neutral species can coexist at physiological pH [39]. The precise molecular mechanism of the antitumor activity of ellipticine is not yet completely known. DNA-binding affinity of the cationic form of the drug is higher than the DNA affinity to the neutral form [40]; however, its biological activity is decreased due to the limited transport across the tumor cell membranes [41]. For preliminary experiments, a sodium acetate buffer of pH 4.5 was chosen as a BGE to ensure positive charge of the ellipticine molecule. Three concentrations of sodium acetate as BGE (10, 50, and 100 mM) were tested (Fig. 2A). The effect was studied with two different concentrations of ellipticine (5 and 30 μM) and the results obtained were compared. As expected, it was found out that the BGE concentration had no significant impact on the migration time of ellipticine in both tested concentrations. On the other hand, there was a marked effect of BGE concentrations on the intensity of ellipticine signal depending on the concentration of the analyte. In the case of 5 μM ellipticine, the highest signal was obtained using 50 mM acetate. In the presence of

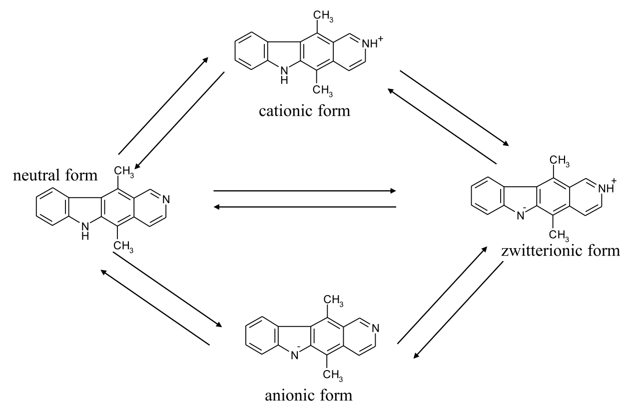

Figure 1. Ellipticine structure and its neutral, ionic, and zwitterionic form. Adapted from [30].

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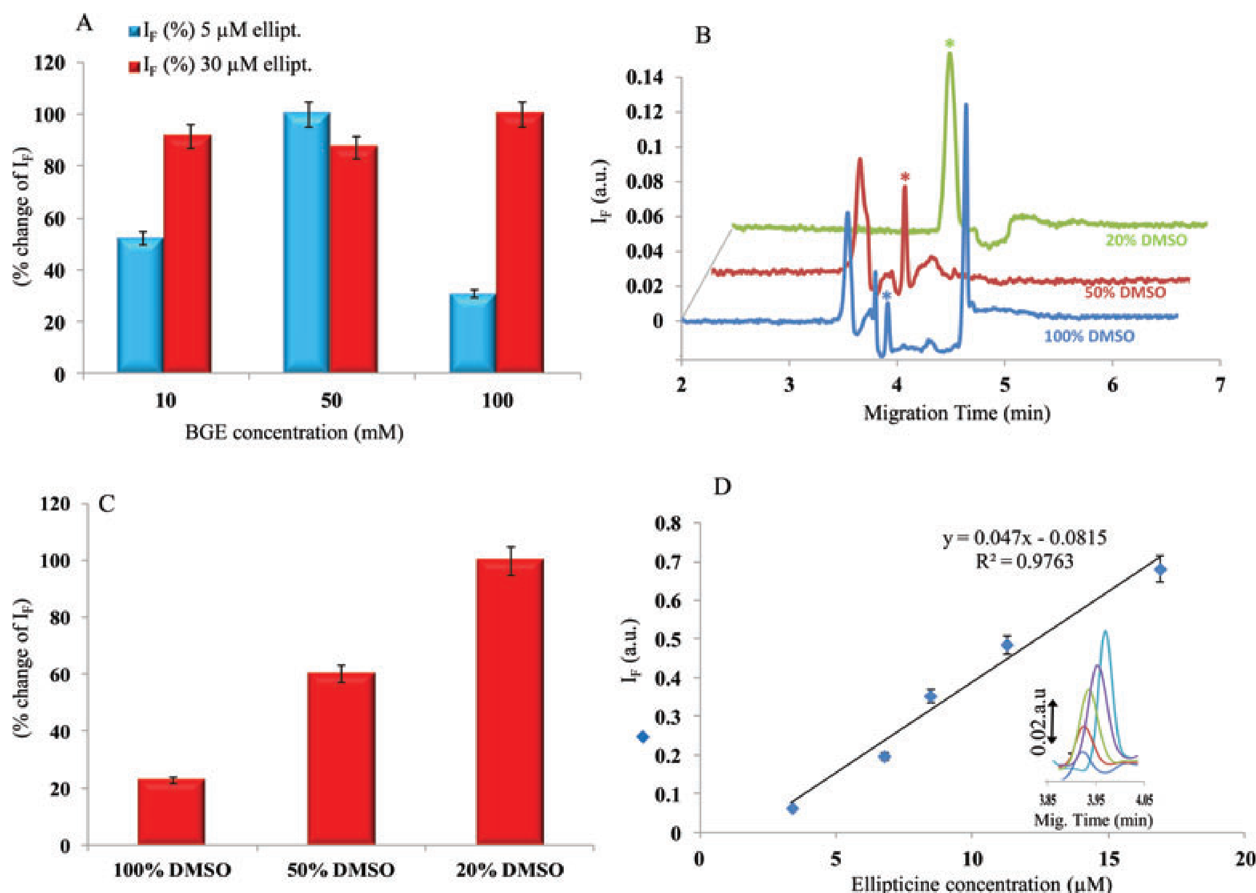

Figure 2. (A) Influence of background electrolyte (BGE) concentration on fluorescence intensity (I_F) and migration time for 5 and 30 μM ellipticine using 10, 50, and 100 mM sodium acetate pH 4.5. Separation conditions: capillary: l_{eff}/l_{tot} = 39/46 cm, id = 75 μm; injection: 20 s, 3.4 kPa; voltage: 20 kV; detection: λ_{ex} 488 nm, λ_{em} 510 nm. (B) Electropherograms of ellipticine (5 μM) dissolved in solvents with different DMSO–water ratio (20%, 50%, 100% DMSO). Separation conditions, same as in Fig. 2A; BGE: 50 mM sodium borate, pH 4.5, peak of ellipticine is marked with *. (C) Dependence of fluorescence intensity and migration time on percentage of DMSO in the sample solvent (data obtained from Fig. 2B). (D) Calibration curve of the ellipticine under optimized conditions. Inset: CE signals of increasing ellipticine concentrations.

higher concentration of BGE (100 mM), the signal decreased by more than 80%. This behavior can be explained the fluorescence quenching caused by the higher ionic strength of the environment. The signal of higher concentration of ellipticine (30 μM) was not influenced by the BGE concentration. This can be explained by the fact that the ellipticine concentration of 30 μM is out of the linear range of the detector response. Therefore, the high concentration self-quenching effect plays more significant role than the quenching caused by increasing ionic strength. Therefore, 50 mM acetate buffer was used for further experiments. It is known that the fluorescence emission maximum of ellipticine is strongly dependent on the solvent. It should be noted that its solubility in water is very poor [42] in comparison to the very good solubility in DMSO. For this reason, ellipticine stock solution has to be prepared in DMSO, but the injection of such solution led to the undesirable effects including the presence of numerous unknown peaks in the electropherogram (ellipticine signal marked with a star in Fig. 2B). It is shown in Fig. 2B that the presence of unspecific peaks was significantly decreased by the increasing water content in the sample solution. This observation is difficult to explain more exactly; however, more importantly, the practical significance of a correct DMSO% in the sample is documented in Fig. 2B. It was found that no additional peaks occurred in the electropherograms of samples containing 20% DMSO. The dependence of the signal intensity as well as the migration time on the sample solvent composition is shown in Fig. 2C. It follows from the results that solvents did not influence the migration time of ellipticine.

Under the optimal conditions as 50 mM sodium acetate as BGE and 20% DMSO as solvent for ellipticine, the dependence of the analyte signal on its concentration was determined (Fig. 2D). The ellipticine signals are shown in inset in Fig. 2D. It clearly follows from the results obtained that migration time slightly increased with the ellipticine concentration. A calibration curve with correlation coefficient (R^2) of 0.9763 was obtained within the concentration range from 3.5 to 20 μM of ellipticine (Fig. 2D). For higher concentrations, the signal height was not linearly dependent on the concentration of ellipticine anymore. The limit of detection (LOD) of ellipticine was estimated as 5×10^{-8} M (signal to noise ratio

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of three). Linear range as well as LOD values are sufficient to study ellipticine interactions with biomolecules, as typical concentrations of this drug applied in clinical practice are few orders of magnitude higher.

3.2 Ellipticine–DNA interaction

Ellipticine exhibits hypochromic and bathochromic changes in absorption spectrum in the presence of DNA. It preferentially binds to helical DNA by intercalation [43]. Biological studies demonstrated that only the protonated form was detected in the cell nucleus due to the increase of pK_a upon DNA binding [44]. CE method optimized in this study was subsequently applied to monitor the interaction of DNA and ellipticine. In the case of ellipticine intercalation into dsDNA, the size and shape of the ellipticine chromophore closely resemble those of a purine–pyrimidine complementary base pair, providing favorable conditions for its intercalation in dsDNA. Furthermore, the polycyclic aromatic character of the molecule may, moreover, result in tight interactions with appropriately conformed hydrophobic regions in DNA. Interactions between the methyl groups of the drug and the thymine bases at the intercalation site appear important in determining the spatial preferences of the drug [45, 46]. To demonstrate the suitability of suggested method for the monitoring of DNA–ellipticine interaction, the mixture of DNA (final concentration of 0.5 mg/mL) and ellipticine (final concentration of 3.5 μM) in 20% DMSO was prepared. This mixture was analyzed in 10-min long intervals. The time dependence of interaction between DNA and ellipticine was monitored (Fig. 3A) showing a distinctive sharp peak (peak 1) with the increasing intensity of the time. It is known that ellipticine primarily interacts with deoxyguanosine in the DNA chain [22, 25]. Therefore, it can be concluded that this peak belongs to ellipticine–DNA complex. After 50th minute of the interaction, a second sharp peak (peak 2) appeared. This peak is believed to belong to the product of intercalation of ellipticine into the DNA structure. It can be concluded that the intercalation is a kinetically slower process in comparison to simple interaction. Dependencies of both peak heights on the time of DNA–ellipticine interactions are shown in Fig. 3B. Dynamics of peak 1 height is higher compared to peak 2, which further support our hypothesis that this peak can be associated with intercalation of ellipticine into the DNA.

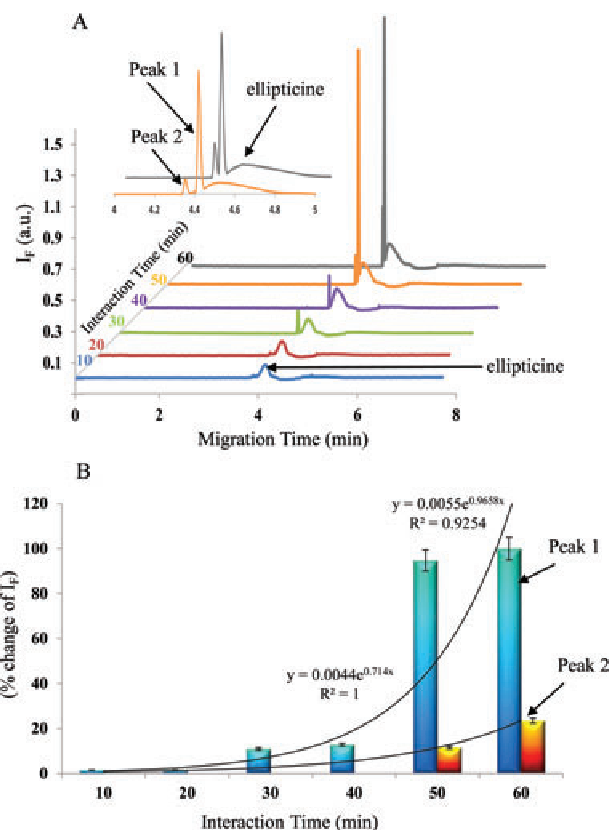

Figure 3. (A) Time dependence of interaction between chicken DNA (1 mg/mL in H_2O) and ellipticine (7 μM in 40% DMSO) mixed 1:1 (v:v). Inset: Detail of DNA–ellipticine complex signals after 50 and 60 min interaction. Separation conditions: capillary: l_{eff}/l_{tot} = 39/46 cm, id = 75 μm; BGE: sodium acetate 50 mM, pH 4.5; injection: 20 s, 3.4 kPa; voltage: 20 kV; detection: λ_{ex} 488 nm, λ_{em} 510 nm. (B) Dependence of DNA–ellipticine complex fluorescence intensity on the time of interaction (peak 1 height–blue, peak 2 height–orange)

4 Concluding remarks

An interaction of drugs with biologically active molecules including DNA is still of great interest. In this study, CE-LIF detection method was found to be useful to study the interactions of DNA with intercalating compound as ellipticine. Two peaks were detected suggesting the presence of various DNA–ellipticine adducts. This method thus represents a new versatile separation-based tool for the ellipticine–DNA interaction studies. Compared to other methods, the optimized CE-LIF proved to be fast and cheap method for the analysis of ellipticine and its adducts with DNA. These adducts are commonly analyzed by ^{32}P-postlabelling technique, but this technique is laborious. Moreover, CE-LIF as one of the very few methods used for ellipticine detection can be miniaturized, which could open new possibilities in the using of lab-on-chip instruments for drug–DNA interactions studies.

Financial support from the following projects CYTORES GA CR P301/10/0356 and CEITEC CZ.1.05/1.1.00/02.0068 is greatly acknowledged.

The authors have declared no conflict of interest.

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