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CE-LIF method for the separation of anthracyclines: Application to protein binding analysis in plasma using ultrafiltration

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Abstract

Anthracyclines are chemotherapeutic drugs that are widely used in the treatment of cancers such as lung and ovarian cancers. The simultaneous determination of the anthracyclines, daunorubicin, doxorubicin and epirubicin was achieved using CE coupled to LIF, with an excitation and emission wavelength of 488 and 560 nm, respectively. Using a borate buffer (105 mM, pH 9.0) and 30% MeOH, a stable and reproducible separation of the three anthracyclines was obtained. The method developed was shown to be capable of monitoring the therapeutic concentrations (50–50000 ng/mL) of anthracyclines. LODs of 10 ng/mL, calculated at an S/N = 3, were achieved. Using the CE method developed, the *in vitro* protein binding to plasma was measured by ultrafiltration, and from this investigation the estimated protein binding was determined to be in the range of 77–94%.

Keywords

Anthracyclines; Capillary electrophoresis; Laser-induced fluorescence; Plasma; Ultrafiltration

1 Introduction

Anthracyclines, including daunorubicin (DAN), doxorubicin (DOX) and epirubicin (EPI), are antibiotics that are regularly used in the treatment of cancers such as lung and ovarian cancers [1]. Both DAN and DOX are active against acute leukaemias, however, DOX has a wider range of activity against solid human tumours such as tumours of the breast, lung, ovary and bladder. It is most effective against soft-tissue sarcomas in adults [2]. EPI, meanwhile, possesses similar response rates to DOX in small cell lung and ovarian cancer. There are less incidences of cardiotoxicity associated with EPI and it is also less myelotoxic [3]. There is an ongoing discussion about the best schedule for anthracyclines, as reduced acute cardiotoxicity was determined with prolonged infusions compared to bolus injections [4]. The dose regimen of anthracyclines is dependant upon the age, size and gender of the patient. The potential for DOX cardiotoxicity is age related and younger children are at a higher risk. The anthracyclines and taxanes, such as docetaxel, are regularly used in combination for the treatment of breast cancer [5]. DOX (40–50 mg/m²) was administered in combination with docetaxel (50–75 mg/m²) to patients with breast cancer and resulted in an overall remission of 71–81% [6]. The use of EPI

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in combination with docetaxel and also in combination with vinorelbine and 5-fluorouracil was also reported in the treatment of breast cancer [7,8].

The determination of free drug concentration is essential, as only the free fraction of a drug is considered pharmacologically active [9]. The extent of protein binding of drugs in biological samples has previously been determined using equilibrium dialysis, ultrafiltration and microdialysis [9–12]. To the best of our knowledge, however, there are only a few reports on the determination of the protein binding of anthracyclines in plasma or serum samples [10, 13–15]

In an investigation by Eksborg *et al.*, the protein binding of some anthraquinone glycosides, including DOX, DAN and EPI, were determined using equilibrium dialysis. The free concentration of anthracyclines was determined using HPLC and their degree of protein binding in serum was reported in the range of 56.9–63.1% [14]. In another application of equilibrium dialysis, the protein binding of DOX in both human and rat plasma was also determined using HPLC. The extent to which DOX was bound to rat plasma was determined to be 78% [10,13]. In addition, using ultrafiltration in conjunction with HPLC the serum protein binding of DAN, DOX and EPI was determined and the degree of binding was reported to vary in the range of 61–78.4% [15].

Separation techniques have been used for a variety of applications, including drug stability, protein binding and therapeutic drug monitoring, such as detailed pharmacokinetic (PK) and metabolic studies [4,10,15–17]. The most prevalent method for their analysis is HPLC, coupled with UV, electrochemical spectrometry, fluorescence spectrometry or MS detection [18–22]. However, recently CE has emerged as a promising separation method as it offers rapid and efficient separations and generates little solvent waste [23–28]. Most importantly, analysis by CE requires only small sample volumes and this is beneficial for the detection of anthracyclines as they are carcinogenic and extremely toxic.

The most common mode of detection for CE analysis of anthracyclines is fluorescence. Fluorescence detection is ideal as anthracyclines possess native fluorescent properties and therefore do not require derivatisation. There are numerous applications of CE coupled with LIF detection for the analysis of anthracyclines [23–25,27,29]. However, to date there is only one CE-LIF method for the separation of DAN, DOX and EPI, the anthracyclines that are under investigation in this report. This method was applied to their analysis in plasma, with LODs in the range of 125–250 pg/mL achieved [23]. In this report, the extent of protein binding of anthracyclines was determined using an ultrafiltration technique. Ultrafiltration may be obtained using either centrifugation or by positive pressure [12], but in this investigation it was achieved by an applied centrifugal force. This is a simple and rapid technique and experiments are performed in a relatively short time [11]. A CE method was successfully developed for the simultaneous separation of DAN, DOX and EPI. From the literature, it is known that these anthracyclines are not administered together; nevertheless, their simultaneous separation is required, due to the similarity in structures of DOX and EPI (they are epimers of each other) and also as DAN is regularly employed as the internal standard. Therefore, the method developed may be applied for either the simultaneous determination of DAN, DOX and EPI or for the individual analysis of either DOX or EPI, using DAN as the internal standard. The determination of bound drug was performed in human plasma samples using ultrafiltration with the developed CE-LIF method. This method was shown to be suitable for the selective protein binding determinations of DAN, DOX and EPI in plasma and it is the first report of the application of ultrafiltration with CE-LIF for their protein binding analysis.

2 Materials and methods

2.1 Chemicals

Boric acid, sodium hydroxide (NaOH), tetradecyltrimethylammonium bromide (TTAB), didodecyltrimethylammonium bromide (DDAB), DAN hydrochloride, DOX hydrochloride, 2-hydroxy-propyl- β -CD, α -CD, methyl- β -CD, sodium chloride, potassium chloride, magnesium chloride, calcium chloride, sodium dihydrogen phosphate and disodium hydrogen phosphate were obtained from Sigma–Aldrich (St. Louis, MO, USA). EPI stock standard was provided by the National Institute of Cellular Biotechnology (NICB), Dublin City University (DCU). Human plasma was purchased from Sigma–Aldrich. Methanol (MeOH) and ACN were obtained from Fisher Scientific (Fair Lawn, NJ, USA). All solutions were prepared in Hydro Nanopure water with a specific resistance of $> 18 \text{ M}\Omega \cdot \text{cm}$ (Labconco, Kansas City, KS, USA).

2.2 CE-LIF separations

All CE-LIF separations were performed on Beckman P/ACE MDQ instrument (Fullerton, CA, USA), equipped with an air-cooled argon (Ar) ion laser at an excitation wavelength of 488 nm. The emission intensities were measured at an emission wavelength of 560 nm (10 nm band width). Polyimide-coated fused-silica capillaries (Polymicro Technologies, Phoenix, AZ, USA) of 75 μm id were employed. The effective length of the capillary utilised was 0.52 m, with a total length of 0.62 m. Sample introduction was performed hydrodynamically (0.5 psi) for 5 s. Data acquisition was performed using a 32 Karat (version 5.0) software. For each standard injection, the capillary was placed into a volume of 50 μL and for the CE separation parameters employed a volume of 25.74 nL was injected onto the capillary.

2.3 CE procedures

All BGEs were composed of a borate electrolyte (105 mM). The BGEs consisted of varying amounts of organic modifiers, ACN or MeOH. The incorporation of various chiral selectors into the BGE were investigated and these were 2-hydroxy-propyl- β -CD, α -CD and methyl- β -CD. The application of MEKC to the analysis of the anthracyclines was studied and the surfactants employed were TTAB and DDAB. The pH of the electrolyte was adjusted to 9.0 using 1 M NaOH. All BGEs were filtered using 0.22 μm Swinney filter prior to use. New capillaries were conditioned with MeOH, 1 M HCl, water, 0.1 M NaOH, water and BGE for 5, 10, 1, 20, 1 and 5 min, respectively. Prior to each injection, the capillary was rinsed for 1, 1 and 5 min with MeOH, 0.1 M NaOH and BGE, respectively.

2.4 Preparation of stock solutions and standards

Stock solutions of DAN, DOX and EPI (2 mg/mL) were prepared in water and stored at 4°C. Stock solutions were prepared fresh every month. Standards of known concentration were prepared by dilution of the stock standards with either 30:70 MeOH/water or 30:70 MeOH/plasma and these were prepared daily due to their poor stability at low concentrations.

2.5 Determination of bound drug by ultrafiltration

Plasma samples (500 μL) were spiked with known concentrations of anthracyclines and centrifuged at $47006 \times g$ and 25°C in a Microcon-Centrifugal Filter Device (Ultracel YM-30, regenerated cellulose 30000 MWCO; Fisher Scientific) for 12 min. The operating parameters employed were specified according to the Fisher Scientific guidelines for these filter devices. The total and free concentrations were determined in triplicate from the sample prior to and after ultrafiltration (*i. e.* the ultrafiltrate), using the developed CE-LIF method. The capillary was changed more frequently when analysing plasma samples, and this was performed when there were noticeable changes in both the detector and current responses. A new capillary was conditioned following the procedure stated in Section 2.3. The volume of bound drug varied

from 29.5 μL for DAN to 113.5 μL for DOX, which are ideal sample volumes for analysis by CE. The bound concentration was calculated from the difference of total concentration less the free concentration.

3 Results and discussion

3.1 Development of CE separation

As stated in the Section 1, the anthracyclines possess native fluorescent properties, due to the presence of a quinone functional group. The structures of DAN, DOX and EPI are shown in Fig. 1. As LIF detection provides 1000 times lower LODs than absorption spectroscopy, it is ideally suited for the therapeutic monitoring of anthracyclines [24,25,30].

In the development of the CE separation, it was determined that the pH of the buffer had a major influence upon the separation. The pK_a values of the anthracyclines vary in the range of 8.08–8.46 [31], and at pH values at least two pK_a units below 8, the anthracyclines will behave as cations. Using a method developed by Reinhoud *et al.*, a phosphate buffer (100 mM, pH 4.2), was applied to the separation of DAN, DOX and EPI. The buffer was modified with an organic modifier, ACN (ACN at 70% v/v □ Verify change made. □), and it was reported to help reduce the interaction of the anthracyclines with the capillary wall, thereby improving the stability and reproducibility of the CE separation, whilst also enhancing their peak shape [23, 32]. However, the separation of DOX and EPI was not achieved with this buffer.

Based upon work reported by Perez-Ruiz *et al.* [24], the application of a borate buffer to their separation was investigated. A pH range of 8–10 was investigated, and it was observed that at a pH of 9.0, the greatest resolution between DOX and EPI was obtained, Fig. 2a. The use of a borate buffer enabled the separation of the epimers, DOX and EPI. This was a direct result of borate complexation. The extent of complexation increases with high pH and is also dependent upon its concentration [33]. In order to improve the stability of the CE separation, ACN was added to the BGE [34]. The incorporation of ACN into the BGE has been reported to help reduce the interaction of the anthracyclines with the capillary wall [23]. The organic modifier also served to increase both the solubility and stability of the anthracyclines.

It was found that adding ACN at 10% v/v into the BGE and sample matrix, improved the peak shapes of all three anthracyclines, in particular the peak shapes of DOX and EPI were considerably enhanced. The separation efficiency (N) of DAN was calculated and it was determined that it had increased from 10000 without ACN to 59450 with 10% ACN present. The anthracycline, EPI, was supplied in a saline solution (0.9% NaCl), and a peak efficiency of 29581 was obtained in its analysis without ACN, as shown in Fig. 2a. Based on the literature, it was known that the addition of ACN in combination with inorganic ions, such as chloride, causes a stacking effect, due to the low solubility of the inorganic ion present and the low conductivity of ACN [34–36]. However, the baseline resolution of DOX and EPI was not achieved with this BGE, and as a result the efficiency of EPI could not be calculated, Fig. 2b. It was also observed that the addition of ACN caused an increase in approximately 2 min in the migration time of all analytes, as shown in Figs. 2a and b. In order to improve the resolution between these analytes, a chiral selector was added to the electrolyte.

The most commonly used chiral selectors in CE are CDs [34,37]. The CDs under investigation in this work were 2-hydroxy-propyl β -CD, α -CD and methyl- β -CD. The concentrations employed in this study were in the concentration range of 1–6 mM, however, the incorporation of these CDs did not improve the separation of DOX and EPI. There are numerous factors which affect the separation of enantiomers such as the volume, diameter and concentration of the CD [34]. MEKC was also applied to their analysis, in order to increase the resolution between DOX and EPI. The surfactants employed in this study were the positively charged

TTAB and DDAB at concentrations ranging from 10 to 50 mM. Nevertheless, the separation of DOX and EPI was not obtained with MEKC.

In order to improve the separation of the target analytes a comparison between the addition of ACN and MeOH in the BGE was performed. Three different BGEs with varying amounts of MeOH (10, 20, 30%) were investigated. In this study, the sample matrices were matched with the BGE under analysis *i. e.* with the BGE containing 10% MeOH the sample matrix consisted of 10% MeOH and 90% water. As mentioned previously, the incorporation of an organic modifier, helped improve their solubility and stability [32]. The presence of MeOH was found to result in an increase in the migration time for all analytes, as shown in Fig. 3a where 30% was used and Fig. 3b where 10% MeOH was used. The peak efficiencies for all analytes using both quantities of MeOH were calculated and are shown in Table 1. From these, it was evident that increased efficiencies and reduced RSD values were determined for DAN and DOX using 30% MeOH, however, for EPI, the efficiency was greatest using 10% MeOH. The stock standard of EPI was supplied in a saline solution (0.9% saline solution) and consequently the increased quantity of MeOH (30%) in the sample matrix resulted in a significant change in the conductivity of the sample matrix when compared to the 10% MeOH solution. Therefore, a reduced efficiency was obtained with the increased quantity of MeOH, as shown in Table 1. Nevertheless, as the addition of MeOH helped improve the resolution between DOX and EPI, aided in an increased response of DAN and DOX and also enabled their therapeutic monitoring, an optimal MeOH content of 30% was employed for further studies.

3.2 Method validation

The analytical method developed was validated with respect to migration time reproducibility with the RSD values of 1.21, 3.54 and 2.05% ($n = 6$) obtained for DAN, DOX and EPI, respectively. The low RSD values obtained indicate the precision of the CE-LIF method. The selectivity of the method was determined through the analysis of six blank sample matrices, with no contaminants or interferences obtained, as shown in Fig. 4. The responses for the anthracyclines were linear in the range of 50–500 ng/mL, with LODs of 10 ng/mL, calculated at a $S/N = 3$, obtained for DAN and 20 ng/mL obtained for DOX and EPI. The RSD values in the range of 2.34–4.12% were obtained ($n = 3$).

3.3 Determination of protein binding using ultrafiltration

The plasma protein binding determinations of drugs have been reported using equilibrium dialysis, microdialysis and ultrafiltration [11,12,38–40]. In this study, ultrafiltration in combination with the developed CE-LIF method, was employed for their protein binding determination. The anthracyclines are highly protein bound, with values reported between 60 and 90% [10,13–15]. It has been reported that during ultrafiltration, the protein concentration changes, which affects the equilibrium constant between the ligand and binding sites and as a result the duration of the ultrafiltration process should be kept as short as possible [12]. However, it has been demonstrated that the concentration of the free drug will remain constant during the ultrafiltration process, provided that the volume of the free drug does not exceed 40% of the volume of the total drug [11]. For this work, the centrifugal speed and the duration of ultrafiltration employed corresponded to the guidelines specified by Fisher Scientific ($47006 \times g$ and 12 min). In a protein binding investigation of anthracyclines by Eksborg *et al.* [14], it was determined that the degree of binding of DAN to albumin was independent of the initial drug concentration employed, and as a result it was assumed that the protein binding of the other anthracyclines were similarly unaffected due to their likeness in chemical structures. In this investigation, the determination of protein binding of anthracyclines was obtained using a concentration of 15 $\mu\text{g/mL}$.

The protein binding studies were repeated in triplicate and the extent to which the anthracyclines were bound to plasma varied from 77 to 94%, and the results are illustrated in Table 2. The results obtained are comparable to those reported in literature, with the low RSDs in the range of 1.68–3.15% obtained [10,13–15]. Figure 4 illustrates a sample electropherogram from a plasma ultrafiltrate of the anthracyclines. It was evident from this that migration time changes did occur, as is evident from the analysis of the anthracycline standards, shown in Fig. 3a. Also, the peak shape of DOX and EPI in the ultrafiltrate had deteriorated. This was a result of the high ionic strength sample matrix, which causes a reduction in the applied field strength, decreases the migration of the anthracyclines and as a consequence band broadening occurs, as shown in Fig. 4 [34]. For these studies, both peak height and area were monitored, and the % protein binding values reported were measured from the peak height, as both parameters resulted in similar protein binding values. Ultrafiltration was previously applied to the serum protein binding of these anthracyclines, binding values in the range of 56.9–63.1% were obtained [15]. The protein binding results achieved in this study were also equivalent to those previously obtained by equilibrium dialysis [10,13,14]. As anthracyclines are highly protein bound, the volume of bound drug will be reduced. In this investigation, the volume of bound drug varied from a minimum of 29.5 μL for DAN to a maximum of 113.5 μL for DOX. These are ideal sample volumes for analysis by CE, as CE only requires nanolitre volumes of sample for analysis. This is the first report of the protein binding determinations of DAN, DOX and EPI using ultrafiltration with CE. In this investigation, ultrafiltration was shown to be an effective, simple and rapid method (12 min) for protein binding determinations.

4 Concluding remarks

A CE method was successfully developed for the separation of the anthracyclines DAN, DOX and EPI coupled with LIF detection. The degree of plasma protein binding of anthracyclines was determined using ultrafiltration and the results obtained were comparable with literature data. Ultrafiltration was shown to be an effective method for the protein binding determination and it is suited to the analysis by CE, as small sample volumes were generated due to their extent of protein binding, and CE only requires nanolitre volumes of sample. The latter is important as exposure to the anthracyclines, which are carcinogenic and extremely toxic, is significantly reduced through analysis by CE.

Abbreviations

DAN	daunorubicin
DDAB	didodecyldimethylammonium bromide
DOX	doxorubicin
EPI	epirubicin
MeOH	methanol
TTAB	tetradecyltrimethylammonium bromide

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Figure 1.

Structures of the anthracyclines, DAN, DOX and EPI.

Figure 2.

(a) CE separation with an excitation wavelength of 488 nm and emission wavelength of 560 nm for the analysis of DAN, DOX and EPI. Concentrations of all analytes 5 $\mu\text{g/mL}$. Separation voltage 17.5 kV, pressure injection 0.5 psi/5 s. BGE: 105 mM borate, pH 9.0. Effective capillary length 52 cm $\times$ 75 μm id. (b) CE separation of DAN, DOX and EPI showing the effect of adding 10% ACN into the BGE. All other conditions as in (a).

Figure 3.

(a) Optimised CE separation of DAN, DOX and EPI. BGE: 105 mM borate, pH 9.0, 30% MeOH. (b) CE separation of DAN, DOX and EPI. BGE: 105 mM borate, pH 9.0, 10% MeOH. Concentration of all analytes 5 $\mu\text{g/mL}$. All other conditions as in Fig. 2a.

Figure 4. CE analysis of (A) of a blank plasma ultrafiltrate and (B) showing the CE separation of DAN, DOX and EPI. The total drug concentration was 15 g/mL. Separation voltage 17.5 kV, pressure injection 0.5 psi/5 s. BGE: 105 mM borate, pH 9.0, 30% MeOH. All other conditions as in Fig. 2a.

Table 1

The separation efficiencies calculated for DAN, DOX and EPI using 10 and 30% MeOH in the BGE and sample matrix

Analyte	N with 10% MeOH (plates/ m)	RSD (<i>n</i> = 3)	N with 30% MeOH (plates/ m)	RSD (<i>n</i> = 3)
DAN	54 490	5.56	72 060	2.25
DOX	22 281	7.71	23 865	0.27
EPI	86 603	5.67	39 234	5.70

All operating conditions as in Fig. 3.

Table 2

The percentage plasma protein binding determined for DAN, DOX and EPI in human plasma

Analyte	Protein binding (%)	RSD (<i>n</i> = 3)	Free drug concentration (µg/mL)
DAN	94.1	1.93	0.89
DOX	77.3	3.15	3.41
EPI	85.3	1.68	2.21

Total drug concentration of anthracyclines was 15 µg/mL. All operating conditions as in Fig. 3.