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Simultaneous analysis of liposomal doxorubicin and doxorubicin using capillary electrophoresis and laser induced fluorescence☆

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ABSTRACT

A method based on a capillary electrophoresis with laser induced fluorescence detection was developed and validated for simultaneous separation of doxorubicin (DOX) and liposomal encapsulated DOX. The separation was accomplished using a fused silica capillary (60 cm in total length, 75 μm I.D.) and potassium phosphate buffer [12.5 mM, pH 7.4] as the running buffer. The effect of sample preparation conditions on maintaining liposomal integrity was also investigated. The limit of detection for DOX was 0.1 μg/ml and the precision and accuracy of CE/LIF method was within the ranges of FDA guidelines. The validated method was successfully used to quantify DOX in human plasma using a direct injection of a 4-fold dilution of spiked liposomal DOX in human plasma.

1. Introduction

Doxorubicin (DOX) is an anthracycline antibiotic that is widely used to treat a variety of cancers such as breast, bladder, lung, ovary, and multiple myeloma [1]. While it is an effective drug, DOX is also quite toxic, and, as with other anti-cancer agents, the clinical toxicities of the agent are often dose and treatment limiting. A unique treatment limiting toxicity associated with DOX and related anthracycline antibiotics is cardiomyopathy which appears in >20% of patients after a cumulative dose of 550 mg/m^2 of DOX [1].

One approach to the reduction of the cardiac toxicity of DOX is the encapsulation of the drug in a liposomal delivery system [2–4]. Two preparations have been reported, one utilizes polyethylene glycol embedded in the lipid layers, pegylated liposomal DOX (Doxil®, Caelyx®) [4]. This formulation has been shown to have significant efficacy in the treatment of metastatic breast cancer and can be safely combined with other anti-cancer agents [4]. The pegylated liposomal DOX was designed to produce prolonged blood levels and this has been associated with both the success of the formulation as well as the observed toxicities, syndrome and myelotoxicity [4].

A second formulation, Myocet®, utilizes non-pegylated liposome and has been shown to be both clinically effective in the treatment of metastatic breast cancer and less toxic with no observed hand–foot syndrome and reduced myelotoxicity [4–7]. The non-pegylated formulation has been shown to be well tolerated and therapeutically active when combined with docetaxel in the treatment of metastatic breast cancer [5] and with paclitaxel and trastuzumab in HER-2-overexpressing breast cancer [6]. The formulation also allows patients who have received the maximum cumulative dose of doxorubicin to receive further doxorubicin, as well as those with risk factors for anthracycline induced cardiotoxicity [7]. The non-pegylated liposomal formulation of doxorubicin represents a potentially important therapeutic advance.

A key aspect of the development of a new drug formulation is the determination of the plasma concentration profiles of the drug. In the case of liposomal DOX, the determination of the pharmacokinetic profiles involved two parallel assays of each plasma sample [8,9]. In one arm of the assay, DOX was assayed after disruption of the liposomes by Triton X-100 detergent followed by solid phase extraction and HPLC analysis. In the second arm, solid phase extraction was used to separate free DOX and liposomal DOX with the assumption that the encapsulated DOX eluted with the applied buffer and the elution of free DOX require chloroform as the eluant. The chloroform eluant was directly assayed by HPLC while fraction containing the liposomal DOX was treated with Triton X-100 followed by solid phase extraction and HPLC analysis.

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The objective of this study was the development of a rapid and direct assay for the simultaneous determination of liposomal encapsulated DOX and free DOX that could be used to monitor the plasma concentrations of free and encapsulated DOX. The analytical technique chosen for this assay was capillary electrophoresis with laser induced fluorescence detection (CE-LIF) based upon the assumption that the charge and size differences between the free and encapsulated DOX would provide the separation and that the native fluorescence of DOX would allow detection of the drug at clinically relevant concentrations. These assumptions were supported by previous reports of the CE-LIF measurements of DOX and DOX metabolites in subcellular fractions [10,11]. The results of this study demonstrate that CE-LIF can be used to directly separate and measure free and liposomal encapsulated DOX in buffer and plasma.

2. Experimental

2.1. Chemicals

The kit used for the preparation of the liposomal encapsulated doxorubicin (Myocet®) was generously donated by Sopherion Therapeutics LLC (Princeton, NJ, USA). Doxorubicin (DOX), potassium phosphate monohydrogen and dihydrogen, fluorescein, boric acid, trizma hydrochloride (Tris–HCl), and tris(hydroxymethyl)aminomethane were purchased from Sigma–Aldrich (St. Louis, MO, USA). Human plasma (pooled) was obtained from Valley Biomedical (Winchester, VA, USA). Sodium chloride for injection (0.9%) was obtained from Cardinal Health (Doublin, OH, USA). Acetonitrile (HPLC grade) was from Fisher Scientific (Pittsburgh, PA, USA). All other chemicals were of the highest purity available. All aqueous solutions were prepared using water from a Millipore Milli-Q water system (Billerica, MA, USA) and filtered using 0.22 μm regenerated cellulose membrane filters purchased from Fisher Scientific.

2.2. Capillary electrophoresis

Capillary electrophoresis (CE) was performed on a Beckman P/ACE MDQ capillary electrophoresis system (Beckman Coulter, Inc., Fullerton, CA, USA) controlled by 32 Karat software (Version 5.0, Beckman Coulter, Inc.). The CE instrument was equipped with laser induced fluorescence detector. The excitation and emission wavelength was 488 and 630 nm, respectively. The emission filter (630 nm) was obtained from Omega Optical (Brattleboro, VT, USA). The electrophoretic separations were performed in a 75 μm I.D. and 60 cm total length (50 cm to the detector window) fused silica capillary column using a running buffer of potassium phosphate buffer [12.5 mM, pH 7.4]. The bare silica capillary was obtained from Polymicro Technologies (Phoenix, AZ, USA). The capillary was cut with capillary cutting tool and a capillary window (0.3 cm) was prepared by burning with flame and removing burned residues. The capillary was filled with 0.1 M NaOH for 3 h and rinsed with water to maximize free silanols on capillary surface. The capillary was again filled with 0.1 M HCl for 5 min and rinsed with water.

2.3. Methods

2.3.1. Liposomal DOX

Liposomal doxorubicin (Myocet®) was prepared according to manufacturers' instruction. Briefly, Myocet DOX HCl (50 mg) lyophilized power was mixed with 20 ml of sodium chloride for injection (0.9%) in a rubber-sealed vial; the mixture was vigorously agitated until fully dissolved and placed on water bath at 60 °C for 12 min. The Myocet liposome (1.9 ml) was mixed with Myocet buffer (3 ml) and the mixture was added to the vial containing the dissolved Myocet DOX HCl. The mixture was vortex-mixed, brought to the room temperature and stored in refrigerator for further use (stable up to 8 h).

2.3.2. Calibration and quality controls

Stock solutions of DOX were prepared by dissolving DOX HCl in running buffer or human plasma at a concentration of 1 mg/ml and subsequently diluted with running buffer or human plasma at concentrations ranging from 0.1 to 100 μg/ml unless stated otherwise.

Calibration and quality controls were prepared daily by spiking drugs to plasma and subsequently diluted with running buffer solution. DOX calibration curves were prepared in the following concentrations: 0.1, 0.5, 1, 5, 10, 50, and 100 μg/ml. One standard curve along with two sets of quality control samples was prepared each day of analysis. The quality control concentrations were as follows: 0.5, 20 and 100.0 μg/ml for low quality control (LQC), middle quality control (MQC) and high quality control (HQC), respectively.

2.3.3. Precision and accuracy

Both precision and accuracy were studied on three different days by analysing QC samples with $n = 5$. Precision and accuracy were also determined for lower limit of quantification (LLOQ) with $n = 5$. The acceptance criteria were taken from the FDA Guidelines [12] where the mean accuracy value for QCs should be within 15% of the actual value except for LLOQ, 20%. Precision determined for each QC level also should not exceed 15% of CVs and 20% for LLOQ.

2.3.4. Stability

The freeze–thaw and short term bench top stabilities of DOX were studied using QC samples. Freeze–thaw tests were performed for three levels of QCs over a 3-day period while bench top stability was studied using low and high QC samples at room temperature for up to 3 h.

3. Results and discussion

3.1. CE separation of DOX and liposomal DOX

The separation of DOX and liposomal DOX prepared in buffer was accomplished using CE. When the optimal separation conditions were employed, the migration time of DOX was ~3.5 min (Fig. 1A), and the migration time of liposomal DOX was ~7 min (Fig. 1B). No interfering peaks were observed in the DOX analysis and a small peak (<10% of total area) corresponding to free DOX was observed in the trace produced by the liposomal DOX solution. It was also observed that for equivalent concentrations of free DOX and liposomal DOX, there was a >5-fold decrease in the RFU produced by the liposomal DOX relative to the free DOX, indicating fluorescence quenching by the liposome. The separation was optimized as described below.

3.2. Optimization of CE separation

Optimization of capillary electrophoretic separation of liposomal DOX and free DOX was performed using different capillary and running buffer conditions. Capillary optimization was performed initially by varying the size of capillary diameter and length. Three different capillary diameters (50, 75 and 100 μm I.D.) with 60 cm in total length capillary were tested. The LIF detection sensitivities, as determined by peak areas, were 100 μm I.D. > 75 μm I.D. > 50 μm I.D. with about a ~2-fold difference between each. However, although the 100 μm I.D. capillary produced the highest detection sensitivity, the electropherogram contained a significant peak corresponding to free DOX relative to the electropherogram observed using the 75 μm I.D. capillary, 10,930 RFU and 4447 RFU, respectively. The DOX peak most probably reflects leakage from the

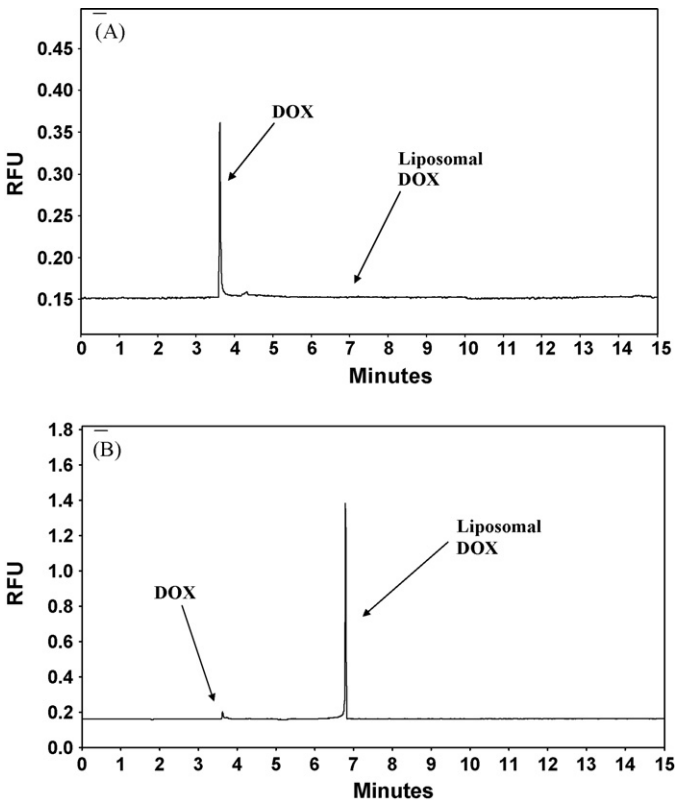

Fig. 1. Representative electropherograms of free DOX and liposomal DOX dissolved in Myocet buffer: (A) free DOX; (B) liposomal DOX (Myocet®); running buffer, potassium phosphate buffer [12.5 mM, pH 7.4].

liposomal DOX due to the higher electrical field strength required with the 100 μm I.D. capillary. Based upon these results, the 75 μm I.D. size capillary was used throughout the rest of the study.

The effect of capillary length on detector response and total analysis time was studied by varying the capillary length from 38 to 98 cm. The migration times of both DOX and liposomal DOX decreased as the capillary length decreased from 10 min (DOX) and 25 min (liposomal DOX) at 98 cm to 3 min (DOX) and 6 min (liposomal DOX) with the 38 cm column. However, significant DOX leaching from the liposomal DOX was observed using the 38 cm column and a total capillary length of 58 cm was selected for further experiments.

The analysis of liposomal doxorubicin was significantly affected by the running buffer conditions such as types of buffer, buffer strength, applied voltage as well as buffer additives. A comparison of potassium phosphate, borate and citrate buffers demonstrated that the potassium phosphate buffer gave the superior efficiency, detector response and analysis time. The concentration of the potassium phosphate running buffer was varied from 1 to 20 mM and the migration times increased with increasing buffer concentration, e.g. DOX migration time increased from 3.7 min (1 mM) to 7.5 min (20 mM) and with the 20 mM buffer there was a 5-fold increase in the amount of DOX leached from the liposomal DOX. The initial studies were conducted using DOX and liposomal DOX dissolved in the Myocet buffer and the optimal running buffer concentration was 12.5 mM. However, when the DOX and liposomal DOX were placed in whole or 50% diluted plasma sample the separation efficiency deteriorated, most probably due to the differences in the electrical field strength between the running buffer and injected solution. It was determined that the separation efficiency and CE performance were stable and tolerable when the amount of plasma was less than 20% of injected solution. Based upon the data from these studies and considering the liposomal integrity, electrical field strength match as well as CE performance, 12.5 mM potassium phosphate buffer was utilized as the running buffer for the remaining studies.

Table 1
The freeze/thaw stability of liposomal DOX (Myocet®) determined as the peak area of free DOX in the electropherogram measured as RFU, where $n = 3$.

Freeze/thaw cycle (number)	Area free DOX (RFU)
0	8,754 ± 113
1	8,686 ± 169
2	8,823 ± 36
3	9,443 ± 474
4	11,553 ± 490
5	14,450 ± 472
6	20,103 ± 771

Previous studies of the CE analysis of DOX have indicated that the addition of acetonitrile to the running buffer increases the efficiency of the assay [10,11]. However, in this study the addition of >10% acetonitrile (v/v) to the running buffer produced a significant amount of DOX leaching from liposomal DOX without significantly improving separation efficiency.

3.3. Freeze/thaw and bench top stability

The freeze/thaw stability of liposomal DOX in Myocet buffer was determined by following the peak area of free DOX, measured as RFUs, observed in the electropherograms over 6 cycles. The data indicated that there was no significant leakage of free DOX over two freeze/thaw cycles, a slight effect after the third cycle and significant disruption after the fourth (Table 1). The results demonstrate that the number of freeze/thaw step should be minimized in sample handling process.

The room temperature bench top stability of liposomal DOX in Myocet buffer was also determined. After a 3-h period, there was less than a 10% increase in peak area of free DOX (data not shown), indicating that the solution was stable under the experimental conditions.

3.4. Analysis of liposomal DOX in plasma

Since the initial studies indicated that the integrity of liposomal DOX was disrupted by as little as 10% acetonitrile, sample extraction techniques such as solid phase extraction and liquid–liquid extraction were avoided. Instead, whole plasma was directly analyzed without pretreatment and the resulting electropherogram contained asymmetric peaks and reduced separation of the free DOX and liposomal DOX (Fig. 2A). It was assumed that the observed results were due in part to the high electrical field generated in the sample plug zone (plasma) compared to the back ground electrolytes. The current (Joule heat) associated with this highly conducting sample plug zone may overcome the capillary thermostating (cooling) capability of the CE system and the excessive Joule heating can have undesirable effects on both resolution and analyte stability. In addition, after whole plasma was injected several times to the same CE column there was significant variability in the migration times and the analysis could not be reliably reproduced. Extensive washing with acetonitrile, 1 M HCl, 1 M NaOH, 0.2 M SDS did not restore the initial migration times. Similar results were observed if plasma was spiked with free DOX instead of liposomal DOX indicating that the capillary had been affected by high concentrations of one or more plasma components. This problem was overcome by a 4-fold dilution of the plasma sample, which restored the efficiency and reproducibility of the separation (Fig. 2B).

Table 2
Precision and accuracy of the CE-LIF method used for the assay of free DOX concentrations in human plasma.

Compounds	Nominal concentration (μg/ml)	Precision		Accuracy	
		Intra-day CV (%)	Inter-day CV (%)	Concentration calculated	RE (%)
Doxorubicin	0.1 (LLQC)	11.4	14.5	0.114	14
	0.5 (LQC)	10.2	11.5	0.482	−3.6
	5 (MQC)	9.8	10.0	5.39	7.8
	100 (UQC)	8.6	7.8	108	8

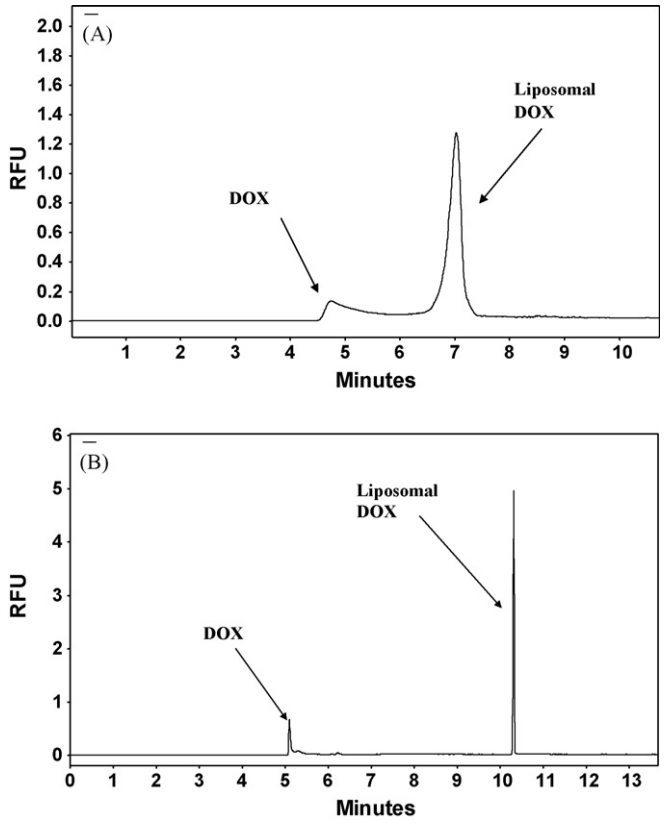

Fig. 2. Representative electropherogram of liposomal DOX (Myocet®) spiked in plasma: (A) whole plasma; (B) plasma sample after a 4-fold dilution with running buffer {potassium phosphate buffer [12.5 mM, pH 7.4]}.

3.5. Validation of the determination of free DOX plasma concentrations

A number of attempts were made to validate the analytical method for the quantification of liposomal DOX concentration is plasma. However, while the measurements were reproducible when the liposomal DOX was dissolved in Myocet buffer, when the liposomal DOX was added to plasma, the assay could not be validated within FDA guidelines [12]. This was observed even after the 4-fold dilution of the plasma samples. It was assumed that this effect was due to the instability of the liposomal DOX formulation (see below). The concentration of the liposomal DOX in plasma and the stability of the liposomal formulation were assessed through the measurement of free DOX plasma concentrations. This approach was selected based upon the assumption that the only source of free DOX in the plasma samples spiked with the liposomal DOX formulation was leakage from the formulation. Therefore, the optimized CE-LIF method was validated for the determination of free DOX.

The calibration curves for free DOX in plasma were linear from 0.1 to 100 μg/ml ($n = 7$) with r^2 values ≥ 0.999. The lower limit of quantification (LLOQ), determined at signal to noise ratio of 10, was 0.1 μg/ml and the upper calibration limit was fixed by the saturation of detector response at >100 μg/ml. The results of accuracy and precision for the CE system are presented in Table 2. The accuracy and intra-day precision were examined by making 20 sequential injections of 5 sets, 4 quality control samples (LLOQ, LQC, MQC and HQC) of a spiked plasma sample. All four sets of QCs showed less than 14% relative errors. The intra-day precision of the system gave precisions of less than 12% at 4 level quality control samples. The inter-day precisions were evaluated over a 3-day period with the analysis of 4 level quality control samples prepared in the same manner as intra-day assay. These gave precision values of less than 15% for DOX.

The bench top, freeze/thaw and post-preparative stabilities of free DOX in human plasma were investigated. The results indicate that free DOX was stable under all of the tested conditions (Table 3).

3.6. Analysis of liposomal doxorubicin in plasma

The plasma concentrations of free DOX were determined using the validated assay. The levels of free DOX were determined 5 min after the addition of liposomal DOX to human plasma at concentrations ranging from 0.4 to 12.5 μg/ml. The 5 min analysis time reflects the time required for sample preparation, capillary preconditioning and analysis. The plasma samples were obtained using serial dilutions of Myocet® that had been prepared following the manufacturer's instruction with a resulting DOX concentration of 2 mg/ml in the liposomal formulation. The studies were conducted at room temperature. The results demonstrated that as the plasma concentration of liposomal DOX increased, the amount of free DOX also increased. However, the relationship between the two concentrations was asymptotic not linear, as the production of free DOX from liposomal DOX approached a plateau at a liposomal DOX plasma concentration of 12.5 μg/ml (Fig. 3). The non-linear, apparently saturable relationship indicates that all or a significant portion of the leakage of DOX from the liposomal formulation was due to an active process, most likely mediated by hydrolytic enzymes such as lipases. In addition, the amount of free DOX derived from a set concentration of liposomal DOX varied with different batches of human plasma indicating the patient to patient variability may also occur due to diverse factors such as age, gender, disease sta-

Table 3
The sample stabilities of free DOX in human plasma in bench top, freeze/thaw and post-preparative studies determined using direct injection of the plasma sample and the CE-LIF assay.

Nominal concentration (μg/ml)	Measured concentration (μg/ml)	CV (%)	RE (%)
3 h bench top			
0.5	0.52	6.9	4.0
100	94.1	11.5	−5.9
Freeze/thaw			
0.5	0.47	7.9	−6.0
100	93.4	12.4	−6.6
Post-preparative			
0.5	0.56	7.4	12
100	97.2	8.9	−2.8

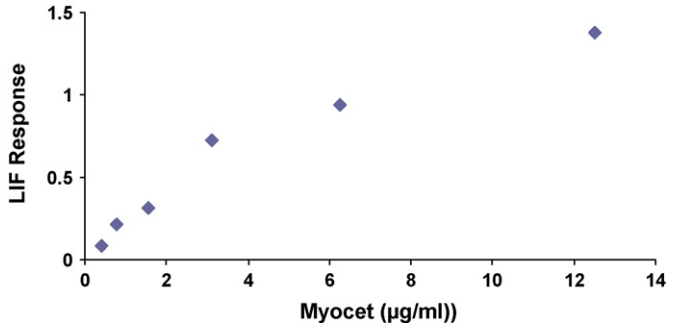

Fig. 3. The relationship of the concentration of free DOX, measured by laser induced fluorescence response (RFU), and the added concentration of liposomal encapsulated DOX in the form of Myocet®.

tus, etc. The validated assay is currently being used to identify the agents responsible for the hydrolysis of the liposomal formulation, the kinetics of this process and to establish if inter-patient variability is of clinical concern. The results of these studies will be reported elsewhere.

4. Conclusions

A CE-LIF separation of free DOX and liposomal DOX has been developed and optimized. The assay was validated for the determination of free DOX in plasma after the addition of the liposomal DOX formulation. The study demonstrated that the key parameters to be considered in the development and optimization of a free drug/liposomal drug assay include capillary dimensions, running buffer strength, applied electric fields and organic modifier concentration in the running buffer. In addition, liposomal DOX in human plasma was analyzed with minimum sample preparation steps, simply the dilution of plasma with the running buffer, minimizing the possibility of the disruption of the liposomal formulation. Since these are relatively standard and easy to address issues, the results of this study indicate that this method should be accessible to a broad spectrum of pharmaceutical and bioanalytical laboratories, and that this approach can be used with other drugs and liposomal formulations.

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