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Abbreviations: BGE: background electrolyte, CE: capillary electrophoresis, DXR: Doxorubicin, UV: ultraviolet, CZE: capillary zone electrophoresis, EOF: electroendosmotic flow, CTAB: hexadecyltrimethyl ammonium bromide, TTAB: tetradecyltrimethyl ammonium bromide, LOD: limit of detection, LOQ: limit of quantification, RSD: relative standard deviation, IEC: ion exchange chromatography, ICP-AES: inductively coupled plasma-atomic emission spectrometry.

Abstract

Optimization and validation of a capillary zone electrophoresis (CZE) method for the determination of inner and external inorganic sulfate from doxorubicin-loaded liposomes as a measurement of encapsulation efficiency is presented. Sulfate was determined using a fused silica capillary (50 cm x 75 µm ID) and background electrolyte (BGE) composed of 5 mM chromate with 1 mM of TTAB at pH 10.5 with indirect UV detection at 254 nm. The separations were achieved at temperature of 25 ºC, 5 kV of reverse applied voltage using a pressure of 0.5 psi for 5s of sample injection. Sulfate quantitation from the outside of liposomes was obtained by simple dilution of the liposome sample but an extraction with a non-ionic surfactant and a mixture of methanol-chloroform-water was necessary to breakdown the liposomes for the quantitation of the total sulfate (inside and outside) with acceptable recovery percentages (96.3% to 102.6%). Parameters of validation were evaluated according to ICH guidelines such as specificity, linearity, LOD, LOQ, accuracy, precision and robustness. The proposed CE method resulted to be suitable for the inner and external sulfate analysis of doxorubicin-loaded liposomes for routine quality and stability control studies.

Introduction

Doxorubicin (DXR) is a potent antineoplastic agent with activity against several human cancer diseases. DXR is a DNA-intercalating agent and a topo-isomerase II inhibitor. It is also known because of its ability to form free radicals which produce lipid peroxidation and can damage DNA, being these effects probably related to its mechanism of action. However, the administration of DXR can produce side effects such as vomiting, bone marrow suppression, alopecia, mucositis and drug/induced dose limiting irreversible cardiotoxicity and myelosuppression.[1-2].

Encapsulation of DXR into pegylated liposomes as drug delivery systems, modifies bioavailability, biodistribution, and reduces side effects. Therefore, achieving high encapsulation efficiency of DXR into liposomes is desirable [1-4].

One strategy to load DXR into liposomes is the use of ammonium – sulfate gradient. The principle is that the charged DXR base diffuses inside the liposome and it is retained within the liposome, and the ammonium diffuses outside [5] (Fig. 1).

One way to measure the efficiency of encapsulation is to determine the concentration of inorganic sulfate, which produces the gradient, on both sides of liposomes membrane.

Inorganic ions analysis is an important area for the role of ions in the pharmaceutical industry. Most pharmaceutical actives are usually charged and they are mainly manufactured linked to a counterion, a metal cation for acid or an inorganic anion for basic drugs [6]. Commonly, such inorganic ions do not have absorbing UV light capacity, therefore different analytical techniques have been developed for liquid chromatography with indirect UV or evaporative light scattering detection [7-9], ion exchange chromatography (IEC) with conductivity detection [10-11], spectroscopic methods such as inductively coupled plasma-atomic emission spectrometry (ICP-AES) [12] and capillary electrophoresis with indirect UV or conductivity detection [13-14].

IEC is the most commonly used analytical methodology for the analysis of inorganic ions in pharmaceutical analysis, but it requires extensive method development and employs different columns, mobile phases and detection modes. Additionally, IEC columns are expensive and require regeneration [5].

Nowadays, CE is a powerful analytical technique used in pharmaceutical analysis due to its high separation efficiency, low sample consumption and the possibility of automatization. Therefore, CE is rapidly gaining popularity as a new highly efficient separation method for analysis of ionic species. Poor UV-response ions can be analyzed by CE-indirect UV detection using a UV absorbing electrolyte. The methods developed by CE with indirect UV allow fast, simple and low-cost quantitation of cations and anions.

To our knowledge, there is no report available in literature about the determination of sulfate in liposome samples by any analytical methodology and capillary electrophoresis has not been specially addressed.

The aim of this work was to evaluate the efficiency of encapsulation of DXR into liposomes as a delivery system through the determination of sulfate ions by capillary electrophoresis. The CE-UV indirect system was based on the use of an UV absorbing electrolyte together with the development of a sample preparation and extraction method for the determination of the total (inside and outside) sulfate content of

the liposomes. CE is a simple and fast method amenable to be applied in quality control and stability studies of liposome formulations.

Experimental

Reagents

Ammonium sulfate, hexadecyltrimethyl ammonium bromide (CTAB) and tetradecyltrimethylammonium bromide (TTAB), were obtained from SIGMA (St. Louis, MO, USA). Extran® MA 02 neutro, potassium chromate, methanol and chloroform were purchased from Merck (Darmstadt, Germany). All chemicals were analytical grade and used without further purification. Ultrapure water was obtained from an EASY pure RF equipment (Barnstead, Dudubuque, IA, USA). All solutions were filtered through 0.45 µm nylon membrane (Micron Separations, Westboro, MA, USA) and degassed before use.
Doxorubicin-loaded HSPC/Chol/mPEG$_{2000}$-DSPE liposomes were kindly provided by Laboratorios Raffo, Argentina.

Instrumentation

All separations were performed with a P/ACE ™ MDQ Capillary electrophoresis system, equipped with diode array detector (190-600 nm) and data were processed by Karat V.8 software (Beckman, Fullerton, CA, USA).
An uncoated fused-silica capillary of 50 cm length (40 cm to detector) and 75 µm i.d. (MicroSolv Technology, Eatontown, NJ, USA) was employed.

Electrophoretic system and capillary conditioning

Sulfate quantitation was performed using a BGE consisting of 1 mM TTAB and 5 mM potassium chromate buffer at pH 10.5. All samples were injected into the capillary by pressure at 0,5 psi for 5 s. The instrument was operated under reverse polarity mode with a constant voltage of -5 kV and detection was performed at 254 nm. Temperature was maintained at 25 °C.
The capillary was rinsed initially with 0.5 M potassium hydroxide for 5 min, then with 0.1 M potassium hydroxide for 5 min, washed with deionised water for 5 min and then with BGE for 5 min. Between runs, the capillary was conditioned with 0.1 M potassium hydroxide for 0.5 min, washed with water for 1 min and then with BGE for 1 min. In all cases, 30 psi of pressure was applied.

Quantitation

Upper phase preparation

Inner sulfate quantitation required the preparation of a solvent mixture consisting of methanol : chloroform : water (1:1:1). After allowing the mixture to stand for some minutes, the upper phase was removed and stored for further use [15].

Preparation of ammonium sulfate standard solution in water

A stock solution containing 1mg mL^{-1} of ammonium sulfate was prepared in water. Solutions for the calibration curve were obtained by appropriate dilution with water in a range of sulfate concentrations from 0.7 µg ml^{-1} to 40 µg ml^{-1}.

Preparation of phosphate, chloride and sulfate standard solution

Individual stock solutions of sodium chloride and sodium phosphate and ammonium sulfate containing 1 mg ml^{-1} of each one of them were prepared in water. Working solutions were obtained by appropriate dilution with water and the final concentration was 12 µg ml^{-1} of each one.

Sample preparation

(A)Quantitation of external sulfate

1 ml of the liposome samples were diluted to a final volume of 25 ml with water.

(B)Quantitation of total sulfate

1 ml of the liposome sample was added to a centrifuge tube, mixed with 250 µl of Extran® MA 02 neutro and vortexed for 1 minute. Then, 3 ml of chloroform, 3 ml of methanol and 3 ml of water were added and vortexed for 1 minute. Afterwards, all tubes were centrifuged at 4000 rpm for 5 min to separate the two phases. The aqueous phase was then removed and transferred to a 25 ml volumetric flask. The remaining phase contained in the centrifuge tube was further mixed with 3 ml of the upper phase solution (see upper phase preparation), vortexed and spinned down as before, and the resultant aqueous phase was collected into the same flask. Extraction step with upper phase was repeated three times, collecting the aqueous phase into the same flask each time. Thus, the whole aqueous phase solution finally collected inside the flask containing the total water-soluble liposomal fractions contains all of the completely recovered sulfate that has been separated from the lipid sample (Fig.2).

Results and discussion

CE-system

Optimization of BGE

CZE with indirect UV detection using chromate as absorbent in the BGE is the most common CE method applied for analysis of anionic compounds.

In BGE, chromate solution pH and cationic surfactant EOF modifier were tested and optimized. Modifications in the pH values from 8.0 to 11.0 generate mobility shifting in anions such as chloride, sulfate and phosphate. At a pH value of 10.5 the baseline was improved and the peaks of the anions had a better shape.

Moreover, the effect of cationic surfactant EOF modifiers such as CTAB and TTAB on the resolution of sulfate and other anions was evaluated. Both EOF modifiers produced no significant change in migration times for inorganic anions at the same concentration of 1 mM; however, TTAB was chosen has modifier because the resultant baseline was more stable during de running.

Optimized BGE consisting of 5 mM chromate solution at pH 10.5 with 1 mM TTAB is showed in figure 3.

Instrumental parameters

Capillary temperature, applied voltage and hydrodynamic injection were optimized to obtain the best conditions. Temperature was evaluated from 20 to 30 °C. High temperatures produced higher ion mobility but less resolution between sulfate and the other anions, whereas low temperatures caused lower ion migration times. Therefore, 25 °C was selected as optimum temperature value of the capillary and sample vials.

The applied voltage was found to be -5 kV for better resolution with suitable current inside the capillary.

Injection time was tested in the range from 1 to 10 seconds and different applied pressure values, being 0.5 psi at 5 s was the best value for obtaining adequate peak symmetry, peak area and resolution of the anions.

Optimization of injection solvent

The influence of dilution media in sulfate UV response was evaluated. Two standard solutions each one prepared in water and the other prepared in UP at the same sulfate concentration were injected six times each one. Results demonstrated that response factor is the same in both cases.

Validation

The validation of the development CE system was accomplished following the International Conference on Harmonization (ICH) guidelines with respect to specificity, precision, linearity, limits of detection (LOD) and quantitation (LOQ), accuracy, and robustness [16].

Specificity

Specificity was evaluated by comparing migrations times of different inorganic ions such as chloride and phosphate considered as interferences for sulfate determination. The resolution factor (Rs) between sulfate and chloride was calculated according to USP 32 [17], the value obtained was 3.0 (Fig 3).

Linearity, LOQs and LODs

Calibration curves at six different concentration levels using standard solutions of ammonium sulfate prepared in water were analyzed in duplicate in three separate runs. The linearity was evaluated in a range from 0.7 µg ml^{-1} to 40 µg ml^{-1}. The LOD (S/R=3) and LOQ (S/R=10) values were 0.1 µg ml^{-1} and 0.4 µg ml^{-1} respectively (table 1).

Precision

Precision was evaluated for intraday assays (n=6) and it was expressed as RSD values for retention times and peak areas (table 1).

Accuracy

Accuracy was tested from recovery studies of samples of ammonium sulfate spikings from their matrix. Sample matrix was simulated using blank liposomes, which were spiked with ammonium sulfate at three different levels (80, 100 and 120%). Three replicate preparations of each level were assayed. Percentage of recovery values in the range between 96.3 % - 102.6 % were obtained (table 1).

Robutness

In order to study robustness, different parameters have been changed in $\pm$ 2% such as chromate solution concentration and pH, time of injection, voltage and temperature. In all cases, no significant changes have been found in migration time and quantitation of sulfate in the real samples.

DXR loaded liposomes samples

The inner and external inorganic sulfate was quantified in DXR-loaded liposomes samples from two pharmaceutical and commercial samples using the proposed method (Fig. 4). The results are shown in table 2.
The quantitation of inner sulfate from the liposomes was optimized using Extran ® MA 02 neutro [18]. The addition of non-ionic detergent resulted in vesicular liposome rupture allowing the extraction of the inner sulfate and consecuently its accurate determination.

Conclusions

The proposed CE-system is considered as the first method to be capable of quantifying sulfate in the presence of other anions in DXR-loaded liposomes.

From the data presented after optimization, validation and quantification of real samples we propose this CE-method as a reliable alternative for the routine analysis of sulfate in DXR-loaded liposomes samples. Advantages of the proposed CE method over the traditional IEC methodologies for the sulfate determination can be obtained in terms of equipment simplicity, low cost of operation, less sample consumption and less waste generation.

In brief, the development of a CE-system is a useful, unexpensive, alternative methodology for quality control purposes.

Acknowledgements

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Legends

Figure 1: Representation of DXR-liposome encapsulation by ammonium sulfate gradient.

Figure 2: Scheme for DXR-liposome sample preparation.

Figure 3: Electropherogram of standard solution of chloride, sulfate and phosphate. Electrophoretic condition see in the text.

Figure 4: a) Electropherogram of sulfate standard solution of (12 µg ml^{-1}), b) Electropherogram of chloride and sulfate standard solution, c) Electropherogram of DXR-liposome sample (total sulfate).

Table 1. Parameter of validation of method of analysis of sulfate

Parameter				
Linearity range [µg ml^{-1}]			0.7– 40	y = 42016x + 212.6
r^2			0.993	
LOD [µg ml^{-1}]			0.1	
LOQ [µg ml^{-1}]			0.4	
Precision (RSD)				
Intraday (n=6)		Migration time	0.1	
		Peak area	2.5	
Interday (n=18)		Migration time	0.3	
		Peak area	2.8	
Accuracy[a]				
	Level	80%	100%	120%
		96.3 (0.6)	99.3 (0.6)	102.6 (0.6)

[a] Percentage recovery mean values obtained from three individual samples on three different days, RSD values in parenthesis.

Table 2. Analysis of sulfate in liposomes from two different laboratories.

Parameter	External sulfate[a] (mg ml^{-1})	Total sulfate[a] (mg ml^{-1})	Encapsuled sulfate[b] (mg ml^{-1})
Laboratory A	0.028 (1.0)	0.523 (0.8)	0.495
	0.029 (1.0)	0.513 (0.9)	0.484
	0.011 (1.0)	0.489 (0.8)	0.478
Laboratory B	0.068 (1.1)	0.607 (0.7)	0.539
	0.063 (1.2)	0.661 (0.7)	0.598
	0.108 (1.0)	0.592 (0.8)	0.484

[a]Results are expressed as mean values (n = 3), RSD values in parenthesis.
[b] Encapsulated sulfate are obtained by difference between total sulfate and external sulfate.

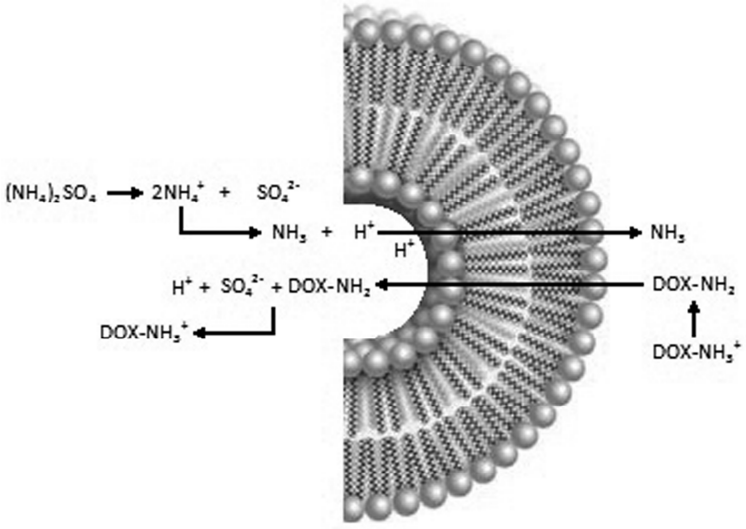

Figure 1: Representation of DXR-liposome encapsulation by ammonium sulfate gradient.

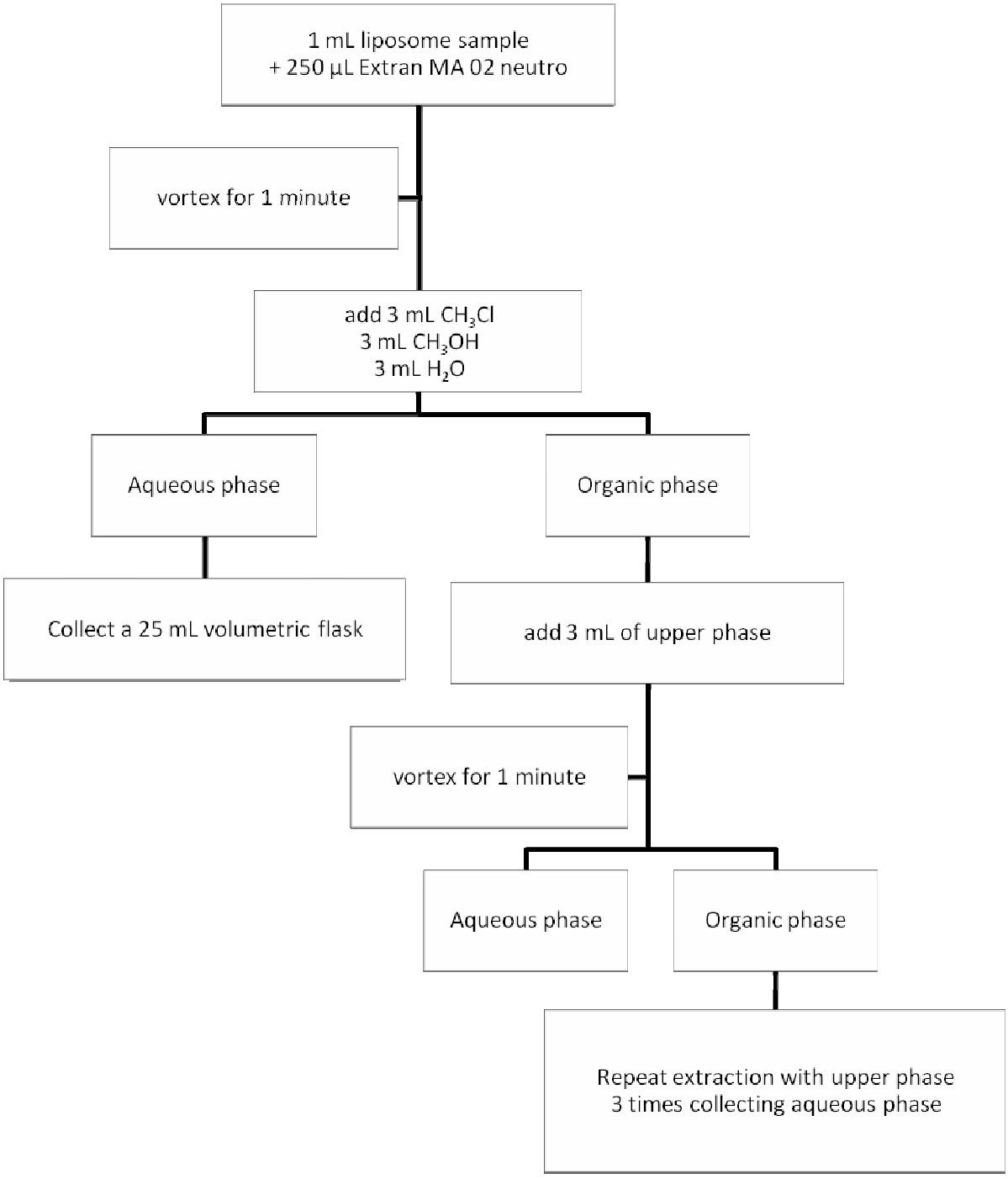

Figure 2: Scheme for DXR-liposome sample preparation.

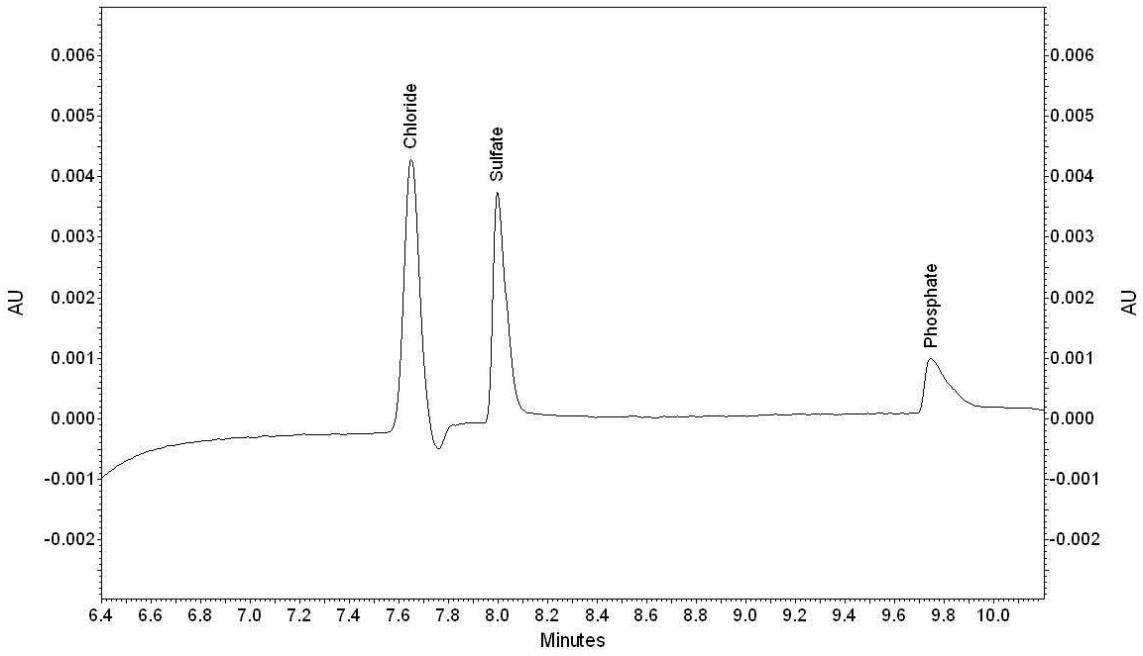

Figure 3: Electropherogram of standard solution of chloride, sulfate and phosphate. Electrophoretic condition see in the text.

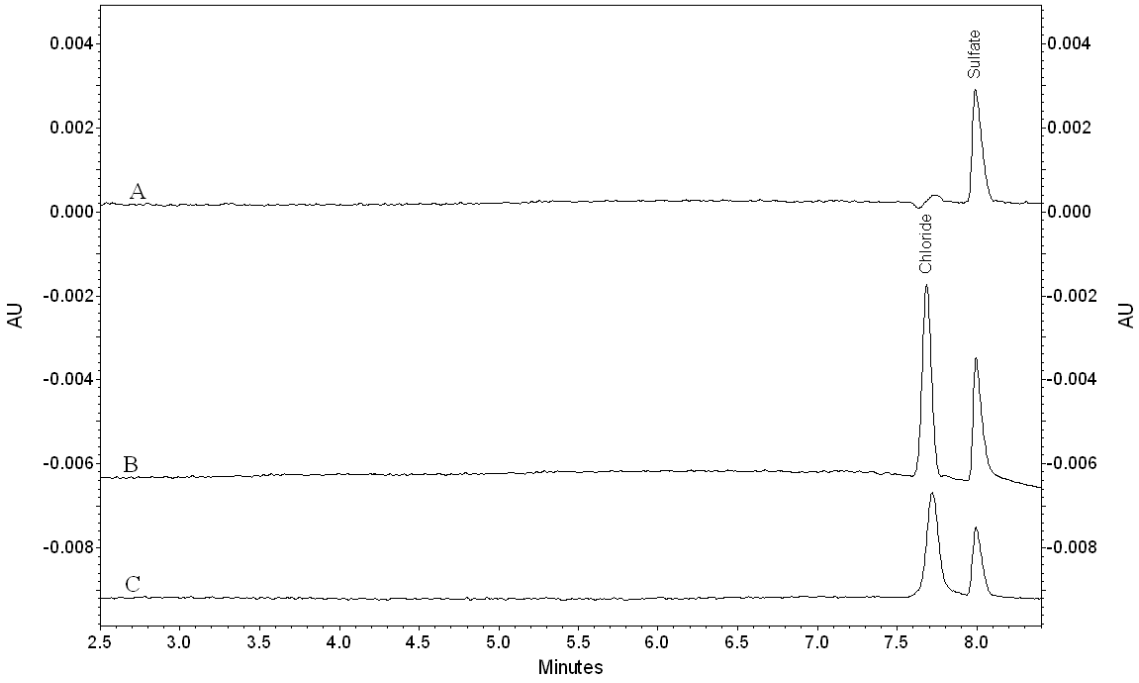

Figure 4: a) Electropherogram of sulfate standard solution of (12 µg ml^{-1}), b) Electropherogram of chloride and sulfate standard solution, c) Electropherogram of DXR-liposome sample (total sulfate).