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Simultaneous determination of doxorubicin, daunorubicin, and idarubicin by capillary electrophoresis with laser-induced fluorescence detection

The separation and simultaneous determination of doxorubicin, daunorubicin and idarubicin was investigated using capillary electrophoresis with laser-induced fluorescence detection. Because the three anthracycline antibiotics were similar in structure and mass, careful manipulation of the electroosmotic flow and electrophoretic mobilities was required. A buffer consisting of 100 mM borate, adjusted to pH 9.5, containing 30% acetonitrile was found to provide a very efficient and stable electrophoretic system for the analysis of the three anthracyclines. The method was applied to the determination of three anthracyclines in serum samples. Responses were linear in the range of 10 – 500 ng·mL^{-1} and the detection limits were lower than 0.9 ng·mL^{-1}.

Keywords: Doxorubicin / Daunorubicin / Idarubicin / Capillary electrophoresis / Laser-induced fluorescence detection
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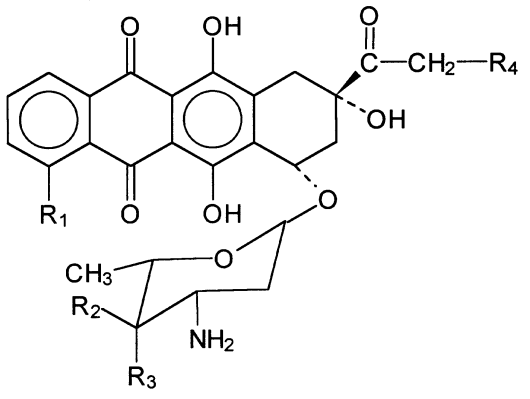
1 Introduction

The anthracycline antibiotics have tetracycline ring structures with a unusual sugar, daunosamine, attached by a glycoside linkage. These antibiotics isolated from several *Streptomyces* species have proton-accepting quinone carbonyl groups and proton-donating hydroxy groups on adjacent rings that permit them to function as electron-accepting and -donating agents. The chemical structures of doxorubicin, daunorubicin and idarubicin are as follows:



	R_1	R_2	R_3	R_4
Doxorubicin (DX)	OCH_3	H	OH	OH
Daunorubicin (DAU)	OCH_3	H	OH	H
Idarubicin (IDA)	H	H	OH	H

DAU and IDA have been used primarily in acute leukemias, whereas DX displays broader activity against human neoplasms, including a variety of solid tumors. The clinical value of these agents is limited by an unusual cardiomyopathy, the occurrence of which is related to the total dose of the drug, and is often irreversible. Only a few methods such as visible spectrophotometry [1], fluorimetry [2], room temperature phosphorescence [3], voltammetry [4], radiolabelled assays [5], thin-layer chromatography [6], and liquid chromatography [7–10] have been proposed for the determination of the anthracycline antibiotics.

Capillary electrophoresis (CE) is a relative new mode of analytical separation with a great potential and is already applied to a wide variety of molecules, ranging from simple ions to large particles, and for ionized as well as for neutral compounds. This technique has already attracted a considerable amount of attention in different areas such as analytical biochemistry, molecular biology, analytical chemistry, and medical biology [11, 12]. Trace analysis at sub-ppb levels can become feasible only if sample preconcentration or improved detection systems are used. Laser-induced fluorescence (LIF) detection is the most sensitive detection system in CE. It is worth noting that to date only four CE assays for anthracycline antibiotics have been reported. DX, DAU and epirubicin [13], and

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Abbreviations: **ADA**, *N*-(2-acetamidol)-2-iminodiacetic acid; **CAPSO**, 3-(cyclohexylamino)-2-hydroxy-1-propanesulfonic acid; **DAU**, daunorubicin; **DX**, doxorubicin; **IDA**, idarubicin; **Tricine**, *N*-tris(hydroxymethyl)methyl glycine

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IDA and idarubicinol [14] were determined in human plasma. DX was also monitored in plasma from pediatric oncology [15] and DAU was analyzed in Kaposi sarcoma tumor [16]. The detection system used was LIF, and the electrophoretic buffer was phosphate buffer adjusted to pH 4 – 5, containing between 30 and 70% v/v acetonitrile.

The aim of this study was to develop a CE method suitable for separating the anthracycline antibiotics DX, DAU and IDA. For this purpose, the pH and composition of the electrophoretic buffer and the proportion of the organic modifier were optimized. The proposed CE method is rapid, sensitive and sufficiently specific for the determination of these antibiotics in serum.

2 Materials and methods

2.1 Reagents and solutions

The hydrochlorides of DAU and IDA were purchased as powders for injection from Pharmacia (Madrid, Spain) and the hydrochloride of DX from Tedec Meiji (Madrid, Spain). Stock standard solutions (500 $\mu g \cdot mL^{-1}$) of each anthracycline were prepared by dissolving the drug in water-acetonitrile (90 + 10, v/v) and stored at 4°C in polypropylene tubes or Eppendorf vials. The addition of 10% acetonitrile was sufficient to avoid adsorption of the anthracyclines to the container walls. Combined standard solutions containing the three antibiotics were freshly prepared from the stock solutions. Borate, phosphate, 3-(cyclohexylamino)-2-hydroxy-1-propanesulfonic acid (CAPSO), CHES, MES, *N*-(2-acetamidol)-2-iminodiacetic acid (ADA) and *N*-tris(hydroxymethyl)methyl glycine (Tricine) buffers were prepared by adjusting to the desired pH with sodium hydroxide or hydrochloric acid. Unless otherwise stated, a 100 mM borate buffer of pH 9.5 containing 30% v/v acetonitrile was used as the electrophoretic buffer. All solutions were prepared using deionized water from a Milli-Q water purification system (Millipore-Iberica, Madrid, Spain), filtered through a 0.45 μm filter (Millisolve Kit; Millipore), and then degassed by sonication and evacuation.

2.2 Apparatus and running conditions

A P/ACE Model 5000 instrument (Beckman Instruments, Palo Alto, CA, USA) with a LIF detector was used for all experiments. The excitation was performed by an air-cooled argon ion laser (3 mW) at a wavelength of 488 nm. The emission intensities were measured at a wavelength of 560 nm filtered by a band pass interference filter (10 nm band width). The instrument was controlled and data were collected with a System Gold data station. Unless otherwise specified, the separation took place in an uncoated fused-silica capillary (57 cm total length; 50 cm to the detector; 75 μm ID) thermostated at 25°C. Sample injection was accomplished by pressure (3.45 kPa) for a time of 5–15 s. The capillary was conditioned every morning before starting a sequence of runs by rinsing in the high-pressure mode for 5 min with 0.1 M hydrochloric acid, 5 min with 0.1 M sodium hydroxide, 5 min with water, and 10 min with the running buffer. After every run it was rinsed for 5 min with the borate buffer. The voltage applied was 15 kV, and the current was *ca.* 40 – 45 μA with the buffer. Excitation and emission spectra were recorded on a Aminco Bowman (Urbana, IL, USA) series 2 spectrofluorimeter.

2.3 Serum samples

The samples were deproteinized by adding acetonitrile. Each serum sample (50 μL) was placed in a test tube and 50 μL of acetonitrile was added. The mixture was vortex-mixed for 1 min and centrifuged for 5 min at 3000 × *g*. The liquid supernatant was diluted to 500 μL with deionized water. This solution was filtered through a 0.45 μm filter before adding to the sample vial.

3 Results and discussion

3.1 Fluorescence measurements

The excitation and emission spectra of DX, DAU and IDA are shown in Fig. 1. As the excitation maximum of the three anthracyclines is between 470 and 495 nm, the use of an inexpensive air-cooled argon ion laser at 488 nm is

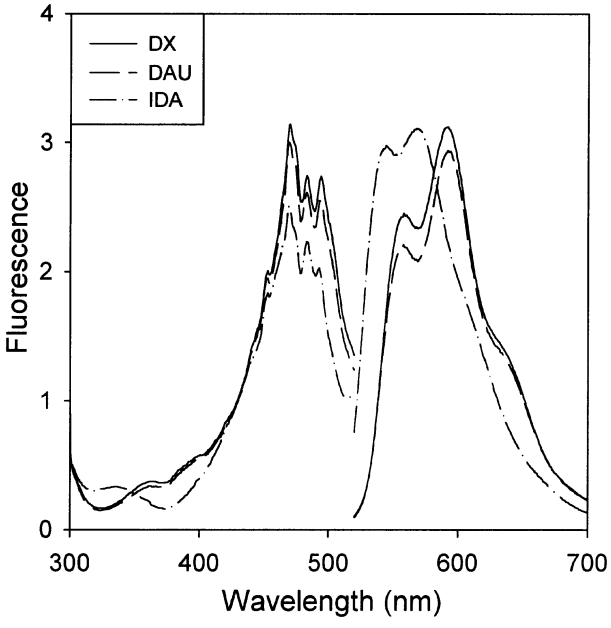

Figure 1. Excitation and emission spectra of the anthracycline antibiotics. Concentration, 2 $\mu g \cdot mL^{-1}$.

optimum for the excitation of these compounds. The fluorescent radiation is measured at 560 nm in order to obtain good sensitivity with all analytes.

3.2 Development of the CE system

The molecular structures of the three anthracyclines provide information about their acid-base properties and about possible ways of separating them by CE. All antibiotics have a sugar amino group (daunosamine) which can be protonated and phenolic groups, which are liable to ionize in alkaline media. In preliminary experiments, it was observed that pH and the type of electrophoretic buffer affected the separation of DX, DAU and IDA. The buffers tested were: borate (pH 8–10), phosphate (pH 3–5 and 6–8), ADA (pH 5.6–7.6), CAPSO (pH 8.6–10.6), CHES (pH 8.3–10.3), MES (pH 5.1–7.1) and Tricine (pH 7.1–9.1). In acid medium, the migration time of the three anthrycyclines was short (< 5 min) because they were present in the form of cations, but it was not possible to separate them. In basic medium, the antibiotics were present in the anionic form and, because their electrophoretic migration velocity is lower than the electroosmotic velocity, they took a long time to migrate (about 7–8 min) when a positive potential was applied across the capillary; furthermore, it was possible to separate them. The borate buffer provided the best results and was selected for all subsequent experiments. The pH of the borate buffer had a strong influence on the resolution of the IDA/DX pair but DAU/IDA resolution was only slightly influenced by pH (Fig. 2). The best separation was obtained at pH 9.5.

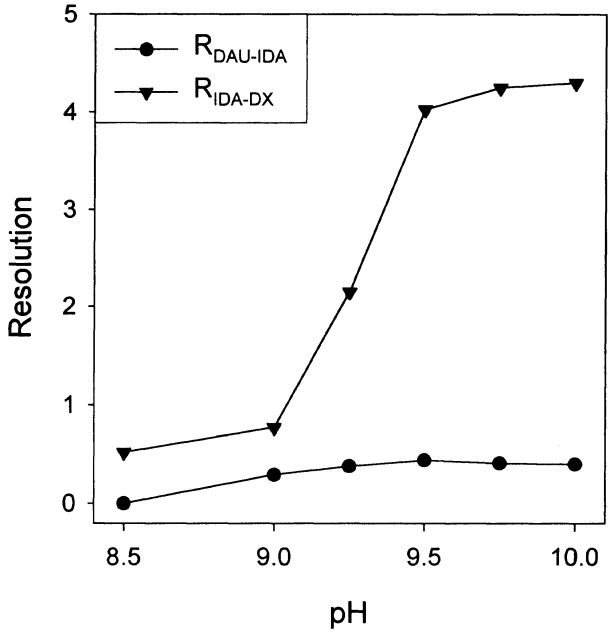

Figure 2. Effect of buffer pH on the resolution of DX, DAU and IDA. Running electrolyte, 100 mM borate.

The presence of acetonitrile, methanol and ethanol lowered the current and increased the migration time of all compounds. Methanol and ethanol slightly improved the separation at the expense of a longer analysis time. However, the addition of acetonitrile to the borate buffer considerably improved the separation (Fig. 3) and caused only a moderate increase in analyte migration time.

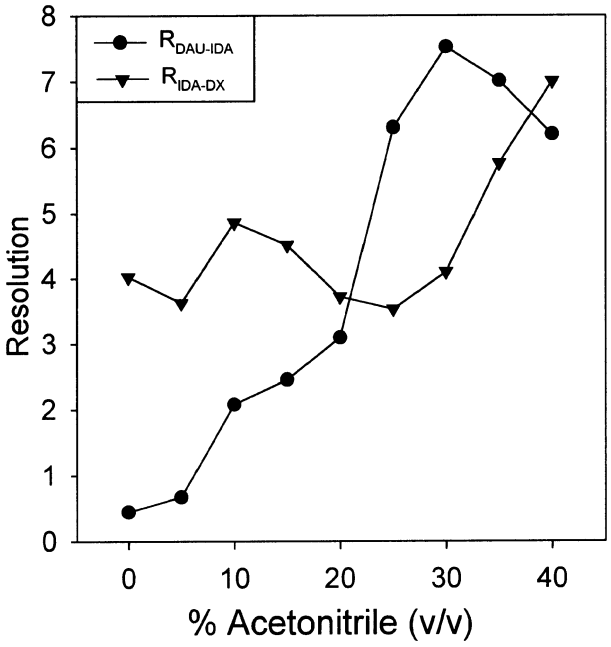

Figure 3. Influence of acetonitrile on the separation of DX, DAU and IDA. Background electrolyte, 100 mM borate buffer (pH 9.5).

The effect of electrolyte concentration on the resolution was studied by increasing the borate buffer concentration from 20 to 150 mM. An increase in the buffer concentration up to 100 mM improved the resolution of the DAU-IDA and IDA-DX pairs. Concentrations higher than 120 mM decreased the separation between DAU and IDA. 100 mM borate buffer of pH 9.5 containing 30% v/v acetonitrile was selected as optimum for the separation of the three antibiotics. To limit the Joule heat generated inside the capillary, the maximum voltages were chosen from the Ohm's law plot. The best-defined peak was achieved with a field strength of 27 kV m^{-1}.

A study was performed to determine the amount of trace enrichment for different injection times and any sacrifice in resolution that resulted. In terms of detectability, high injection volumes are favorable, but the resolution decreases as a result of the broader zones. It was possible to perform pressure injections of up to 15 s with no loss in resolution but with a satisfactory linearity of peak area against injection time (analyte quantity). It was ob-

served that injection times larger than 20 s resulted in poor peak areas because of disturbance of the sample-buffer interface zone.

3.3 Analytical performance characteristics

Under the conditions providing optimum separation, the samples can be analyzed quantitatively. Table 1 gives the results of their statistical evaluation using an injection time of 5 s. The calibration graph performed with nominal concentrations of the solution standards against measured peak areas showed good linearity over the range of 4–500 ng mL^{-1}. The migration of the anthracycline antibiotics was very reproducible, the RSD being within the range of 0.3–0.4%. The detection limits were estimated through the IUPAC model modified for chromatography [17] using a longer injection time (15 s). The values summarized in Table 1 show the high sensitivity of the method.

Table 1. Analytical data for the CE determination of the anthracycline antibiotics (peak area, arbitrary units, *versus* concentration, ng·mL^{-1})

Compound	A[a)]	B[b)]	(r^2)[c)]	LOD[d)]
DX	160.5 ± 1.9	–34.9 ± 16.4	0.9995	15
DAU	224.3 ± 2.0	–42.4 ± 17.4	0.9998	10
IDA	392.5 ± 3.5	–20.9 ± 9.5	0.9998	8

a) Slope of the regression lines fitted to the calibration data set ± standard deviation
b) Intercept of the regression lines fitted to the calibration data set ± standard deviation
c) Correlation coefficient of the calibration graph
d) Detection limit in pg·mL^{-1}

Reproducibilities in the peak area measurements were studied by injecting eleven replicate solutions containing 25 ng·mL^{-1} of each analyte. The peaks corresponding to the anthracyclines (see Fig. 4) are completely separated and the peak areas for DX, DAU and IDA were constant for ten consecutive runs with RSD_s of 2.3, 2.5 and 2.4%, respectively. When the same sample was run 30 times over a seven-days period, the RSD_s were 5.8, 4.1 and 5.6% for DX, DAU and IDA, respectively.

3.4 Determination of DX, DAU and IDA in serum

Preliminary experiments about the linearity and precision of the assay for serum using spiked samples revealed that there was linearity in the range of 10–500 ng·mL^{-1}. The correlation coefficient of the regression lines was 0.999 or higher. The precision of the method was assessed by determining three concentrations within the range of 25–250 ng·mL^{-1} in three independent series of samples; RSD_s of below 4% were always obtained. Day-to-day precision data were obtained over a period of five working days. Table 2 summarizes the results obtained for the three antibiotics in serum samples. Figure 4 shows the electropherogram of a serum sample spiked with 25 ng·mL^{-1} of each anthracycline; the clean background obtained was due to the combination of the sample preparation procedure and the selective detection.

Table 2. Analytical recovery and precision of the CE method for serum samples

	Concentration added (ng·mL^{-1})	Recovery (mean ± SD)	RSD (%) Intraday	RSD (%) Interday
DX	25	24.5 ± 0.8	2.3	3.0
	100	96.0 ± 3.7	2.2	5.9
	250	248.4 ± 4.7	2.7	6.6
DAU	25	24.0 ± 1.0	2.3	6.3
	100	100.7 ± 1.7	1.0	2.9
	250	236.6 ± 3.5	2.1	6.5
IDA	25	24.9 ± 1.8	3.2	6.2
	100	103.2 ± 2.2	1.2	3.8
	250	235.4 ± 4.3	2.5	3.9

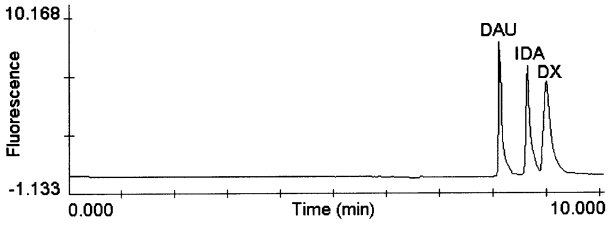

Figure 4. CE separation of the anthracycline antibiotics in spiked serum samples. Analyte concentration, 25 ng·mL^{-1}. The electrophoretic buffer was 100 mM borate (pH 9.5), containing 30% acetonitrile. Separation tube, 57 cm × 75 μm ID fused-silica capillary.

The LOQ of DX, DAU and IDA in serum based on a signal-to noise ratio of 10 are 3.6, 4.0 and 7.2 ng·mL^{-1}, respectively. These values are not low enough for pharmacokinetic studies and so a longer injection time (15 s) and a less diluting sample treatment were used. The LOQ values now obtained were less than 0.9 ng·mL^{-1}. The usefulness of the method was also demonstrated with the analysis of plasma samples from two cancer patients treated with 50 mg·m^{-2} of DX and 12 mg·m^{-2} of IDA. The results obtained are shown in Table 3.

4 Concluding remarks

We have described a novel CE method with LIF detection for the quantitative determination of DX, DAU and IDA in human serum, which is specific, accurate and precise. The sample pretreatment procedure is based on a rapid

Table 3. Concentration of DX and IDA in serum samples from treated patients[a)]

	Drug treatment ($mg \cdot m^{-2}$)	Serum concentration found ($ng \cdot mL^{-1}$)
DX	50	20.4
IDA	12	23.0

a) Samples were obtained from two female patients with ovarian cancer after 4 h of an intravenous infusion dosage

precipitation step. Compared to previously described assays for anthracycline antibiotics, the proposed method provides equivalent and even superior sensitivity. The LOQ in serum was 0.9 $ng \cdot mL^{-1}$ for DX, 0.8 $ng \cdot mL^{-1}$ for DAU and 0.6 $ng \cdot mL^{-1}$ for IDA. The method was applied to therapeutic drug monitoring in clinical samples.

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