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Short communication

Anthracycline analysis by capillary electrophoresis Application to the analysis of daunorubicine in Kaposi sarcoma tumor

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Abstract

Laser-induced fluorescence (LIF) detection is now a well-known sensitive and selective detection mode for capillary electrophoresis (CE) analysis. It has been shown to be 100- to 10 0000-times more sensitive than UV detection and little work has been done using LIF in conjunction with high-performance liquid chromatography (HPLC). The need for greater resolution and higher sensitivity for the analysis of anthracyclines (fluorescent chemotherapic drugs), prompted us to compare CE–LIF and HPLC–LIF, for the detection of these substances. CE–LIF sensitivity based on quantity of anthracycline injected is 50-times greater than that obtained with HPLC–LIF, because of the injected sample volume. Analysis of daunorubicin in Kaposy sarcoma tumors and in plasma are presented. The decrease of the concentration of daunorubicin in the tumor and in the plasma following time show the same behavior, indicating identical concentrations of the anthracycline in both samples. © 1999 Elsevier Science B.V. All rights reserved.

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1. Introduction

Reverse phase high-performance liquid chromatography (HPLC) and capillary electrophoresis (CE) are two of the most important techniques for analytical scale separations of a large variety of samples. A large number of studies were done using laser induced fluorescence (LIF) detection with CE dealing with nucleic acids, proteins, peptides, amino acids, inorganic and organic cations or anions [1]. This kind of detection did not have much success when combined with HPLC, when looking at prostaglandins [2], fatty acids [3] or steroid hormones [4]. In fact, the very small injection volume of CE (some nanoliters), imposes the use of such a sensitive detection mode for very diluted samples. The injection volumes in HPLC are at least 1000-times greater. For the same sample, the number of molecules detected is 1000-fold higher in HPLC than in CE. Yet, the first LIF detection systems were first made for capillary HPLC [5] but their success was quite limited.

Gassman et al. [6] used the Folestad et al. LIF detection system [5] on a capillary for CE. Some years ago, we developed a new kind of LIF detector using collinear detection with a ball-lens for collimating the laser beam on the capillary [7]. This

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system allows a very easy focalization of the laser beam on the capillary, which can be quickly adjusted by the user. Its high numerical aperture allows very sensitive detection. We demonstrated earlier that $3.5 \cdot 10^{-13}$ M of rhodamine 123 could be identified [7].

The anthracyclines, doxorubicin and daunorubicin are the most widely used antimelanoma agents, with a broad spectrum of activity against a variety of malignancies. These drugs are believed to exercise their cytotoxic action through a number of different mechanisms [8]. The relative contribution of their different mechanisms to cell death are at present unknown [9]. However, in vitro studies have demonstrated a relationship between cellular anthracycline levels and cytotoxicity [10]. Pharmacokinetic investigations in patients treated with anthracyclines have so far given inconclusive results. The possibility of monitoring cellular anthracycline, at very low concentrations, would be a great help for further elucidation of the relationship between anthracycline pharmacokinetics and the therapeutic outcome [11].

Doxorubicin and daunorubicin have been detected and quantitated using standard HPLC techniques [12–14] with UV or conventional fluorescence detection. Polarography [15], high-performance thin-layer chromatography [16] and CE [17] have also been used to identify these anthracyclines and some of their derivatives. Reinhoud et al. used CE–LIF to identify some anthacyclines in plasma [18]. Very recently Hemple et al. described a clinical study of doxorubicin using CE–LIF with a 15 mW argon ion laser [19]. To our knowledge, the best level of sensitivity is the detection of 50 fmol in reversed-phase HPLC using conventional fluorescence [12] and 48 amol using CE–LIF and a 1 W Argon ion laser [18].

In our work, we wanted to model the intensity of fluorescence in capillaries of different inner diameter with high numerical aperture optics. An optimum diameter of 250 μm I.D. was chosen, which is compatible with our HPLC flow-rate (0.5 ml/min). Then, we compared the use of HPLC–LIF and CE–LIF for quantitation of doxorubicin, with our LIF ball-lens detector. We attempted to do a study on daunorubicin in Kaposy sarcoma biopsies using CE–LIF, which is less sample consuming than HPLC–LIF.

2. Experimental

2.1. Instrumentation and separation conditions

A modular injector and high-voltage power supply Prime Vision 100 (Europhor Instruments, now Picometrics, Toulouse-Ramonville, France) equipped with a modular LIF detector for capillary (Zeta Technology now Picometrics) and a 488 nm wavelength laser (532 MBS, 10 mW or 20 mW, Omnichrome, Chino, CA, USA) were used. The detector is equipped with the 488 nm filter set (Picometrics), including a 488 nm dichroic mirror, two 488 nm notch filters, a 515 nm high pass filter, and a 1.5 mm spatial filter. A 95 cm (effective length 42 cm)×75 μm I.D. fused-silica capillary (Polymicro Technologies, Phoenix, AZ, USA) was used. The separation buffer consisted of acetonitrile–50 mM sodium dihydrogenophosphate buffer, pH 4 (2:8, v/v). The capillary was rinsed with 0.1 M NaOH for 3 min, with water for 2 min, and then with separation buffer for 3 min. Samples were injected by hydrodynamic injection (reduce pressure of 375 mBar) for 3 s (100 nl). Separations were carried out by applying a separation potential of 20 kV resulting in an electrophoretic current of 61 μA. HPLC studies were run with an isocratic system (Eurosas, Toulouse, France) equipped with a 5-μl injection loop, a 15 cm length Econosphere C_{18} column (Altech, Les Ulis, France) and a constant 0.5 ml/min flow-rate. For the LIF studies, a fused-silica 250 μm I.D. capillary is fitted at the output of the column. We chose a distance of 10 cm between the column and the detection window on the capillary.

All Chemicals were purchased from Aldrich. (St Quentin Falavier, France).

For the comparison between CE–LIF and HPLC–LIF, we used the 20 mW Ar ion laser.

For comparison between HPLC–LIF and HPLC conventional fluorescence detection, a Shimadzu fluorescence detector RF10AXL (Shimadzu, Duisburgh, Germany), excitation 488 nm, emission 580 nm was used as conventional fluorescence detector. The Zeta LIF detector was fitted with the 20 mW laser tube.

2.2. Biopsy treatment

Biopsies and plasmas came from patients having Kaposi sarcoma, who were treated with 60 mg/m^2 doses of daunoxome (encapsulated daunorubicin) [20]). Biopsies and plasmas were taken from patients at different times following injection of daunoxome.

The 0.1 g of biopsies (around 10^8 cells) were treated with a lysis buffer for degradation of DNA and proteins (2.5 ml collagenase, 2.5 ml 0.5% trypsin, 100 μl 0.1% DNAse). One hundred μl of 25 n*M* rhodhamine 123 solution was added to the samples as well as an internal standard. The daunorubicin extraction was made using 0.9 ml, 0.05 *M* borate buffer, pH 9.8 and 3 ml of dichloromethane. This volume was vortexed for 1 h, and centrifuged for 20 min at 1500 *g*. The upper layer was removed and dried. Before CE analysis we added 0.5 ml of acetonitrile–water (1:1, v/v).

The calibration graph for biopsy studies: 10^8 sarcoma cells were incubated 6 h with 1 ml daunorubicin 1, 4, 7, 10, 25 and 60 n*M* solutions. 0.6 ml of the lyse buffer was mixed with cells, sonicated and incubated 1 h at 37°C. The sample was then treated like the biopsies. CE–LIF was run using an 20 mW Ar ion laser.

The calibration graph for plasma analysis: 1 ml of non-pathological plasma was added with 1 ml daunorubicin 1, 4, 7, 25 and 60 n*M* solutions. Samples were treated like biopsies without the lyse buffer step. A modality scale was used to compare concentrations following time of daunorubicin in plasma and tumors.

3. Results and discussion

3.1. Comparison between CE and HPLC performances, and LIF and conventional fluorescence

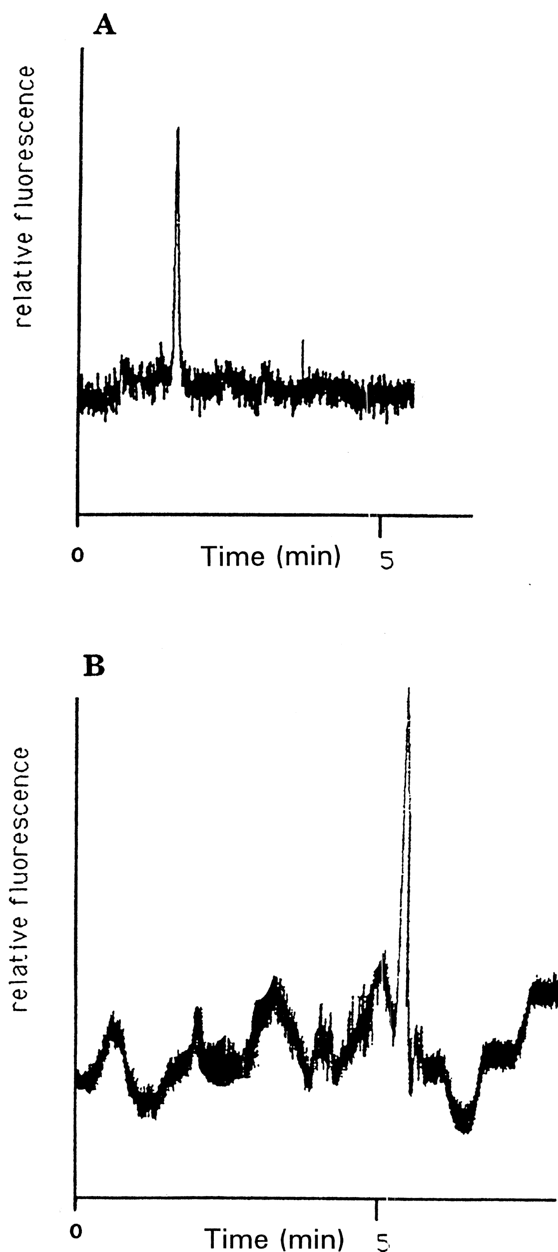

Fig. 1. Analysis of a $5 \cdot 10^{-11}$ *M* doxorubicin solution diluted in water detected with LIF detection. (A) Analysis with HPLC, (B) analysis with CE.

Fig. 1 shows that we have the same molar sensitivity in CE–LIF and in HPLC–LIF, limited to $5 \cdot 10^{-11}$ *M* with a signal-to-noise ratio of 8. Nevertheless, the quantity of doxorubicin injected is more important in HPLC (5 μl, 250 amol) than in CE (100 nl, 5 amol). This could be due to the very high resolving power of CE that allows one to have a large number of theoretical plates and to concentrate the sample in a very small volume. Moreover the flat

shape of the electroosmostic flow contribute to limiting diffusion [21]. The linear response of the detector is appreciated by plotting the log of concentration of doxorubicin following the log of the fluorescence. We obtained a straight line between concentrations of 10^{-8} *M* and 10^{-10} *M*. The slope α of the curve for CE is $\alpha=0.98$ and $\alpha=0.99$ for HPLC. These values, very close to 1, show a good linearity of the detector response [22].

Fig. 2 is a comparison of $1.8\cdot10^{-9}$ *M* doxorubicin and daunorubicin solution analyzed by HPLC with conventional fluorescence (A) and with LIF detection (B). LIF detection is considerably more sensitive than conventional fluorescence detection. The signal-to-noise ratio of the conventional fluorescence detector we used was $S/N=11$, whereas it is higher than 500 for LIF detection.

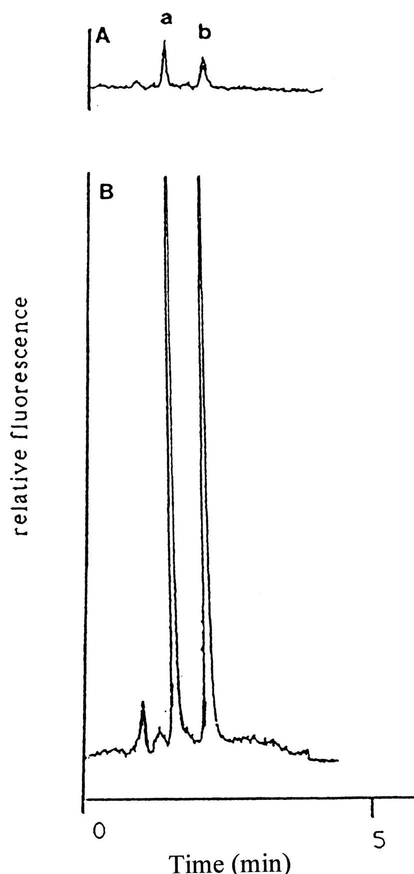

Fig. 2. Comparison of a 5-μl injection of a $1.8\cdot10^{-9}$ *M* doxorubicin (a)/daunorubicin (b) (1:1) mixture separated with HPLC using (A) conventional fluorescence detection, (B) LIF detection. The chromatograms are presented with the same noise intensity.

The possibility to analyze, using CE, a microliter sample several times, and the great sensitivity of LIF detection allowed us to analyze the decrease over time of daunorubicin extracted from Kaposi sarcoma tumors.

3.2. Application to the evolution of the concentration of daunorubicin in Kaposi sarcoma tumor by CE–LIF

Fig. 3A represents a plot of the log of fluorescence as a function of the log of peak area, on two-orders of magnitude (10^{-7}, 10^{-9} *M*) after the extraction procedure for biopsies and plasmas. The slope β of the curve is $\beta=0.81$ for biopsies and $\beta=1.02$ for plasmas. β for biopsies is far from a slope of 1.00 and indicates that the extraction of daunorubicin does not give a constant yield following the different concentrations. It could be due to adsorption of the anthracycline on the laboratory material used during the extraction steps. Fig. 3B shows an electropherogram of an extract of daunorubicin in a biopsy 18 h after the injection of this anthracycline in the patient. The extracted sample was diluted 10-times before analysis. The intra-day variability calculated as the RSD of daunorubicin in plasma is lower than 0.6% for plasmas and 9.7% for tumors ($n=4$, for each concentration). The use of an internal standard greatly increase the reproducibility especially at low concentration. Fig. 3C shows the decrease of the molality (mol/kg) of daunorubicin in the tumor and in the plasma following time. Approximately the same decrease is observed in both biological samples. Showing that the behavior of the concentration of the anthracycline is identical in the blood and in the tumor, the daunorubicin is getting inside the tumor. The comparison of the slopes of the two kinetic curves indicates a slight retention of the anthracycline in the tumor compare to the plasma.

4. Conclusions

CE or HPLC associated to LIF can be used to study anthracyclines in biological samples. The molar sensitivity is identical, but molecular sensitivi-

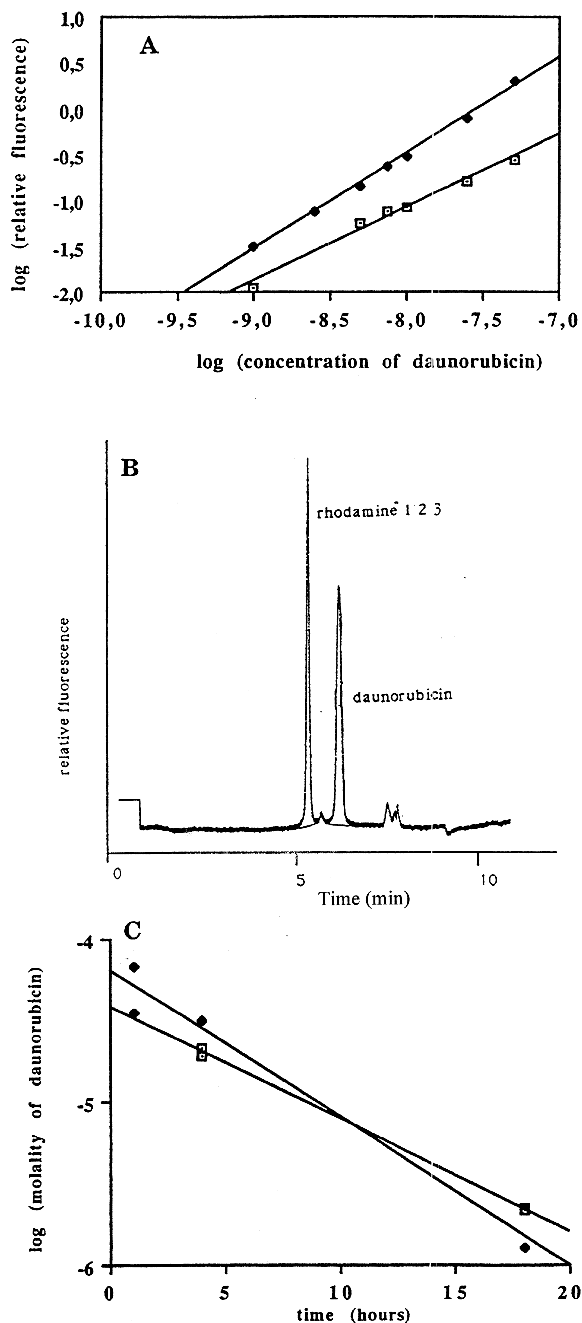

Fig. 3. Analysis of daunorubicin in Kaposi sarcoma biopsies. (A) Calibration curve of concentration of daunorubicin in plasmas (♦) and in tumors (□) after extraction steps, and using rhodamine 123 as an internal standard. (B) Electropherogram of an extract of daunorubicin in a Kaposi sarcoma biopsy (18 h after injection), the extracted sample was diluted 10-times before the CE–LIF analysis. (C) Kinetic study of daunorubicin decrease following time in Kaposi sarcoma tumors.

ty is greatly in favor of the use of CE, which is fifty time more sensitive. This technique will be preferable when the collected samples from the patient are very small (some microliters) or in the case where sample must be concentrated before the analysis, whereas HPLC could be used when one uses higher sample volume. Moreover, we showed the very high sensitivity level of LIF detection prior to conventional fluorescence detection with an HPLC separation system. Using CE–LIF, we were able to monitor the kinetics of daunorubicin release in Kaposi sarcomas. The decrease of the concentration of daunorubicin in the tumor and in the plasma following time shows the same behavior, indicating identical concentrations of the anthracycline in both samples.

References

[1] H.E. Schwartz, K.J. Ulfelder, F.-T.A. Chen, S.L. Pentoney, J. Cap. Electrophoresis 1 (1994) 36.
[2] V.L. McGuffin, R.N. Zare, Proc. Natl. Acad. Sci. USA 82 (1985) 8315.
[3] J. Shin Yoo, V.L. McGuffin, J. Chromatogr. 627 (1992) 87.
[4] J. Gluckman, D. Shelly, M. Novotny, J. Chromatogr. 617 (1984) 443.
[5] S. Folestad, L. Johnson, B. Josefsson, Anal. Chem. 54 (1982) 925.
[6] E. Gassman, J.E. Kuo, R. Zare, Science 230 (1985) 813.
[7] G. Nouadje, M. Nertz, Ph. Verdeguer, F. Couderc, J. Chromatogr. A 717 (1995) 335.
[8] J. Cummings, N. Willmott, J.F. Smyth, Eur. J. Cancer 27 (1991) 532.
[9] P.A.J. Speth, Q.G.C. van Hoesel, C. Haanen, Clin. Pharmacokinet. 15 (1988) 15.
[10] P.A.J. Speth, R.A.P. Raijmakers, J.B.M. Boezeman, C. Haanen, Eur. J. Cancer Clin. Oncol. 24 (1988) 667.
[11] B. Sundman-Engberg, U. Tidefelt, J. Liliemark, C. Paul, Cancer Chemother. Pharmacol. 25 (1990) 252.
[12] A. Andersen, D.J. Warren, L. Slørdal, Cancer Chemother. Pharmacol. 34 (1994) 197.
[13] A. Andersen, D.J. Warren, L. Slørdal, Ther. Drug Monit. 15 (1993) 455.
[14] M.J. Sepaniak, E.S. Yeung, J. Chromatogr. 190 (1980) 377.
[15] S.M. Golabi, D. Nematollahi, J. Pharm. Biomed. Anal. 10 (1992) 1053.
[16] A. Farina, M.G. Quaglia, A. Doldo, S. Calandra, F.R. Gallo, J. Pharm. Biomed. Anal. 11 (1993) 1215.
[17] M.G. Quaglia, A. Farina, F. Kilar, S. Fanali, E. Bossu, C. Dellaquila, J. Liq. Chromatogr. 17 (1994) 3911.
[18] N.J. Reinhoud, U.R. Tjaden, H. Irth, J. van der Greef, J. Chromatogr. 574 (1992) 327.

[19] G. Hemple, P. Schulze-Westhoff, S. Flege, N. Laubrock, J. Boos, Electrophoresis 19 (1998) 2939.
[20] P.S. Gill, B.M. Espina, F. Muggia, S. Cabriales, A. Tulpule, J.A. Esplin, H.A. Liebman, E. Forssen, M.E. Ross, A.M. Levine, J. Clin. Oncol. 13 (1995) 996.
[21] J.W. Jorgenson, K.D. Luckas, J. Chromatogr. 218 (1981) 209.
[22] R.P.W. Scott, in: Liquid Chromatography Detectors, Journal of Chromatography Library, Vol. 11, Elsevier, Amsterdam, 1977, Ch. 2.