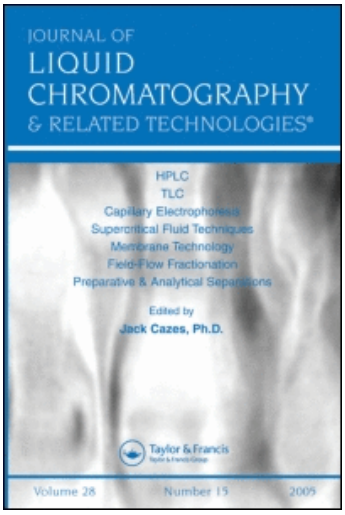

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ANALYSIS OF A NEW DOXORUBICIN DERIVATIVE (FCE 23762) AND RELATED COMPOUNDS BY HIGH PERFORMANCE CAPILLARY ELECTROPHORESIS

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ABSTRACT:

High performance capillary electrophoresis was applied to analyse FCE 23762, a new antracycline analogue (doxorubicin derivative) with anti-tumor activity. It has been shown earlier that a FCE 23762 sample may contain two main impurities, one of the optical isomers of FCE 23762 and the most probable degradation product, adriamycinone. The pharmacologically active compound and its diastereomer were resolved in free zone electrophoresis experiments. Adriamycinone, a neutral compound, is baseline separated from the other two (cationic) compounds by micellar electrokinetic chromatography. The use of two different separation methods was advantageous for an efficient and sensitive analysis for the synthetic product of FCE 23762.

INTRODUCTION

Anthracycline antibiotics are chemotherapeutic agents with significant anti-tumor activity. Besides doxorubicin and daunorubicin a large number of analogue compounds have been synthesized with the aim to find anti cancer drugs with improved efficacy and minor toxicity. The clinical use of doxorubicin and other related compounds in cancer diseases, e.g., acute leukaemia, lymphoma and solid tumor, has been approved (1-5). The emergence of the resistance against these drugs observed in experimental conditions as well as in patients, however, initiated further research to develop (synthesize) new analogues. Among those, the class of morpholino anthracyclines and particularly a new compound, 3'-deamino-3'-[2-(S)-methoxy-4-morpholino]-doxorubicin (laboratory code FCE 23762), maintained good effect on doxorubicin-resistant tumor cells (6). FCE 23762 is a derivative of doxorubicin with a modified daunosamine moiety in position 3' synthesized and studied in the Chemical Research and Development Department of Farmitalia - Carlo Erba (Milan, Italy).

Figure 1. shows the structures of 3'-deamino-3'-[2-(S)-methoxy-4-morpholino]-doxorubicin (**I,** code: FCE 23762), 3'-deamino-3'-[2-(R)-methoxy-4-morpholino]-doxorubicin (**II,** named in this paper as "R-isomer") and adriamycinone (**III**). The "R-isomer" might appear as synthetic impurity in a FCE 23762 sample together with adriamycinone which is the most probable degradation product of both, FCE 23762 and "R-isomer".

The high toxicity and easy degradation of the doxorubicin derivatives require sensitive and selective analytical methods to perform stability tests and drug monitoring in biological fluids. HPLC techniques have been used widely for the analyses of anthracyclines (7-13), and among them two specific methods have been developed for stability studies of FCE 23762 and for its determination in

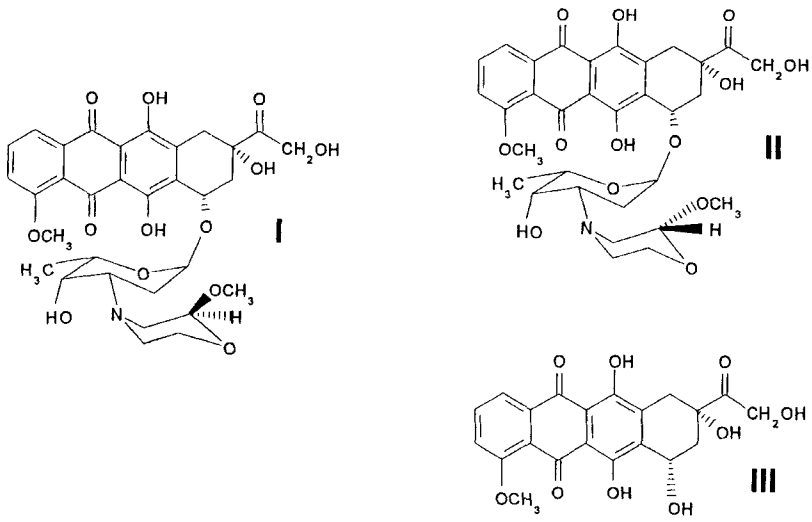


FIGURE 1. Chemical structures of (**I**) 3'-deamino-3'-[2-(S)-methoxy-4-morpholino]-doxorubicin, (**II**) 3'-deamino-3'-[2-(R)-methoxy-4-morpholino]-doxorubicin and (**III**) adriamycinone.

plasma (11,12). Some of the HPLC methods (13), however, showed low efficiency.

High performance capillary electrophoresis (HPCE) has also been employed to analyse daunorubicin, doxorubicin and epirubicin in human plasma (14). Since HPCE is a promising new separation method, providing high efficiency and sensitivity within short analysis time, we applied capillary electrophoretic methods for the determination of FCE 23762 and its related compounds.

EXPERIMENTAL

Chemicals

Samples (reference standards) of FCE 23762, "R-isomer" and *adriamycinone* were kindly supplied by the Chemical Research and Development Department of

Farmitalia - Carlo Erba (Milan, Italy). The purity of the FCE 23762 sample (considered as reference standard), attested by the Department, was: 3'-deamino-3'-[2-(S)-methoxy-4-morpholino]-doxorubicin 90.0 %, water 7.0 %, 3'-deamino-3'-[2-(R)-methoxy-4-morpholino]-doxorubicin 1.6 %, adriamycinone 0.4 % and unknown impurities 0.8 %.

Double distilled water was used for the separation of the electrolyte and the sample solutions. All common chemicals (acetic acid, sodium hydroxide, acetonitrile, etc.) were purchased from Merck. The solutions were degassed and kept at room temperature during the working day.

Stock solutions of the samples were prepared in water and diluted to the appropriate concentration before the electrophoretic analyses.

Capillary electrophoresis

Capillary zone electrophoresis (CZE) was performed in sodium phosphate (pH 2.5, 5.0, 6.0 or 7.0) or sodium acetate buffer (pH 4.2 or 5.0) without or with CH_3CN, as organic additive. Micellar electrokinetic chromatography (MEKC) experiments were made in a 15 mM phosphate - 6 mM borate buffer (pH 7) containing 20 mM SDS.

The analyses were carried out with a Spectra Phoresis 1000 Instrument (Thermo Separation Products, San José, CA, USA), equipped with a multiwavelength UV-VIS detector. Separations were performed in a CElect FS75 (75 μm ID) bare fused silica capillary (Supelco, Bellefonte, PA, USA) with a total length of 42 cm and effective length of 34 cm. Capillary conditioning were done every day (washing with 0.1 M NaOH for 30 min) and before every runs (washing with 0.1 M NaOH for 2 minutes, with water for 2 min and with background

electrolyte for 3 min). The samples were applied with vacuum for 1.5 s or 2 s (ca 3 nl/s). The temperature of the capillary cartridge was kept at 15 °C in the zone electrophoresis and 25 °C in the micellar electrokinetic chromatography experiments.

The SpectraPhoresis 1000 equipment was controlled and the data were evaluated with the SpectraPhoresis CE v.1.05B software (Thermo Separation Products). The samples were identified either with spiking or with the help of their spectra obtained by the multiwavelength detection.

RESULTS AND DISCUSSION

The purpose of this study was to obtain a fast capillary electrophoresis method to analyse the homogeneity and purity of FCE 23762, a new doxorubicin derivative with anti-tumor activity. In the synthetic procedure of FCE 23762 two other compounds might cause inhomogeneities, the "R-isomer", and a degradation product, adriamycinone (see Fig. 1). We have investigated the effect of separation mechanism (zone electrophoresis and micellar electrokinetic chromatography), pH and organic additive (modifier) in the background electrolyte on the resolution of the above three compounds.

The samples obtained from Farmitalia - Carlo Erba were considered as reference standards. At the same time, however, they were also regarded as "unknown" samples to be characterized by electrophoretic runs, because it was attested by the producer, that FCE 23762 sample contains also "R-isomer" and adriamycinone as impurities. The other two samples were not characterized in details by the supplier. In the electrophoretic experiments, therefore, mixtures of the samples (containing high amount of each compounds) were used to find

optimal conditions for the separations, but afterwards the stock solutions of the samples were investigated in the conditions, where the components had been best resolved.

FCE and "R-isomer" migrate in electric field as cations, while adriamycinone is a neutral compound. The separation of the two diastereomers, FCE 23762 and the "R-isomer", does not need, in principle, a chiral selector because they have different physico-chemical properties and therefore they could migrate with different electrophoretic mobilities. For resolving these compounds we used zone electrophoresis experiments in different buffer systems, such as, sodium phosphate at pH 2.5, 5, 6 or 7 and sodium acetate at pH 4.2 or 5.0. For the improval of the resolution different concentrations of acetonitrile, as organic additive, were tested.

Zone electrophoretic experiments, performed at acidic pH, showed, that an organic additive, acetonitrile, was always necessary for obtaining the resolution of the two diastereomers. This is probably due to the changes in the solvatation of the two diastereomers caused by the organic modifier which may influence the effective mobilities of the substances. Higher concentration of the acetonitrile, however, caused longer experimental runs (i.e., longer migration times of the substances in the same background electrolyte). In the phosphate buffer at pH 2.5 it was not possible to obtain baseline resolution of the two diastereomers, even an acetonitrile concentration as high as 70 % was applied (results not shown). Fig. 2a shows the baseline separation of FCE 23762 and "R-isomer" in 50 mM sodium acetate pH 4.2 in the presence of 70 % acetonitrile. Increasing the pH to 5.0 the acetonitrile concentration could be lowered to 30 % (Fig. 2b). Further increase of the pH was not useful, since the high electroosmotic flow (EOF) did not allow the separation of the two components because they were moving with the EOF (results not shown). Adriamycinone (neutral substance) in a mixture of all three

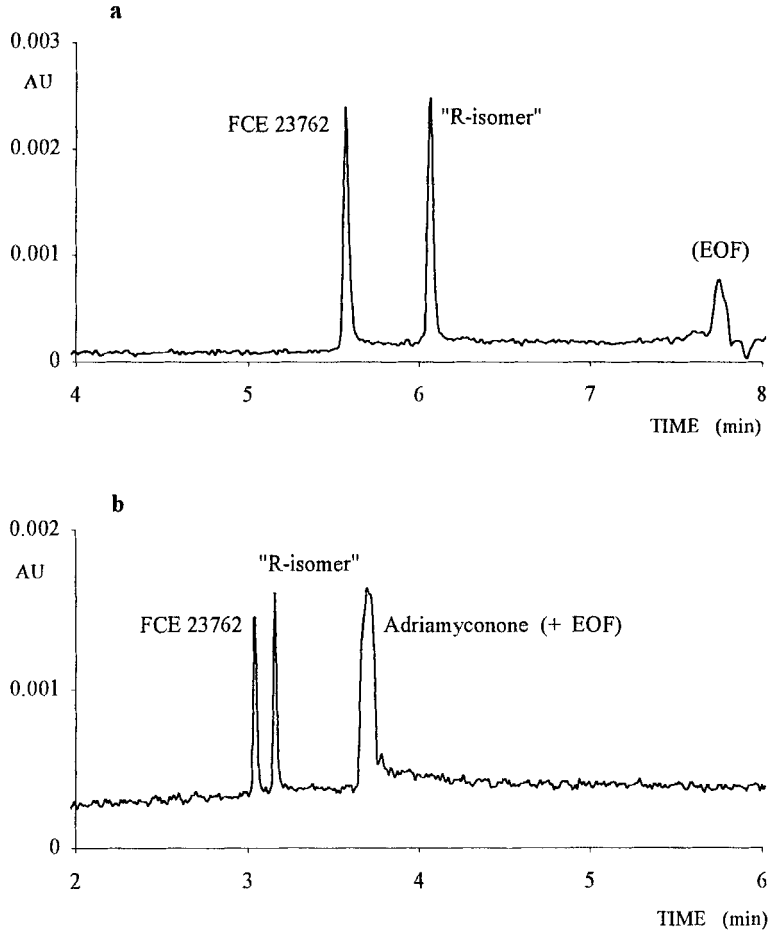


FIGURE 2. Capillary zone electrophoresis analyses of sample mixtures of (a) FCE 23762 (10 μg/ml) and "R-isomer" (10 μg/ml) and (b) FCE 23762 (5 μg/ml), "R-isomer" (5 μg/ml) and adriamycinone (8 μg/ml). Background electrolyte (a) 50 mM Na-acetate, pH 4.2, containing 70 % acetonitrile, (b) 50 mM Na-phosphate, pH 5.0, containing 30 % acetonitrile. Experimental conditions: capillary 75 μm ID x 42 cm (34 cm effective length); voltage 20 kV; current (a) 16 μA, (b) 30 μA; detection 225 nm; temperature 15 °C. Sample application was made by vacuum for 1.5 s. (EOF, electroosmotic flow).

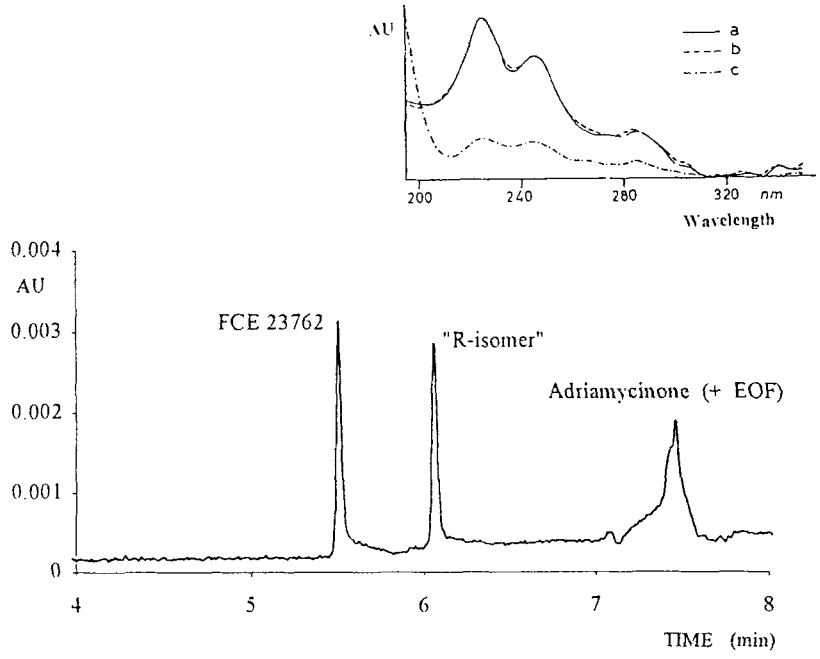


FIGURE 3. Capillary zone electrophoresis of a mixture of FCE 23762 (10 µg/ml; insert: a) "R-isomer" (8 µg/ml; insert: b) and adriamycinone (7 µg/ml; insert: c). Background electrolyte and experimental conditions are as in Fig. 2a. (EOF, electroosmotic flow). The spectra were obtained with multiwavelength detection.

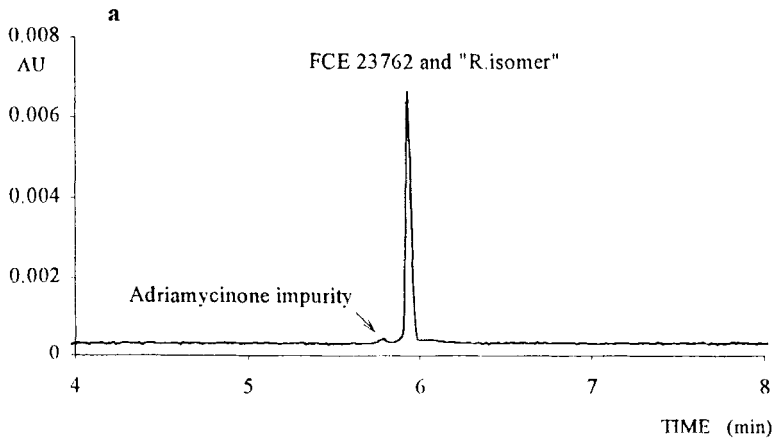
compounds of interest (see Fig. 2b and Fig. 3) moved with the EOF forming an asymmetric peak.

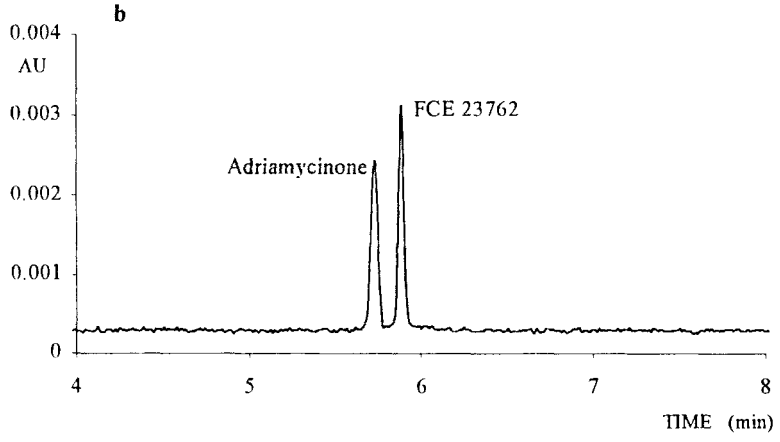
For the separation of adriamycinone we used micellar electrokinetic chromatography in a phosphate-borate buffer containing 20 mM SDS. The two diastereomers of interest are not resolved in this condition (Fig. 4a), however, both, FCE 23762 and "R-isomer" were baseline separated from adriamycinone (Fig. 4b,c).

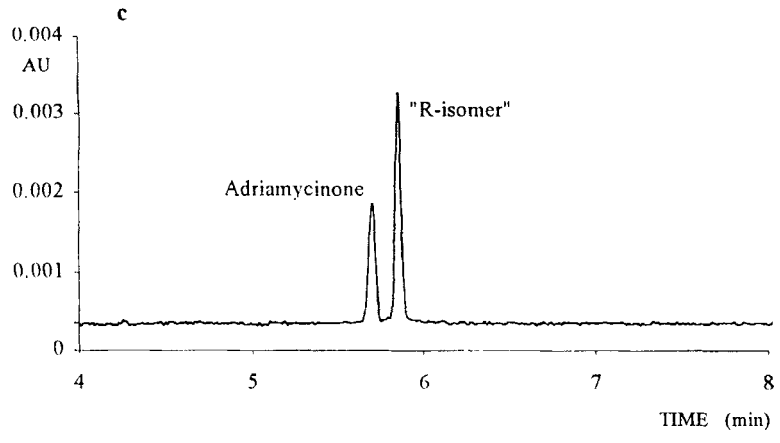
With the above two electrophoretic methods we analysed the original samples, as well. Using the experimental conditions in Fig. 2 and 3, the minimum detectable amount of "R-isomer" was obtained to be 0.5 % of the amount of FCE 23762 (using) in a mixture of both (the experimental error of the determination of the peak area ratio was found to be ± 0.3 % due to the baseline variations, probably caused by the influence of the organic additive or the other impurities). As it was expected from the purity testing attested by the producer, the FCE 23762 sample contained a small amount of the "R-isomer" (1.6 % of the sample that corresponds to ca. 1.8 % of the FCE 23762 content). The amount of the "R-isomer" was estimated from the electropherogram of the FCE 23762 stock solution (not shown) and found to be 2.0 % ± 0.3 % of the FCE 23762 content, which is in a good agreement of the previous characterization. However, the MEKC experiments did not show detectable amount of the adriamycinone in the sample (Fig. 5a), which means that our method has a detection limit of the related compound ca. 0.4 % of FCE 23762 or higher. Unexpectedly, the sample of the "R-isomer" showed the presence of adriamycinone detectable in the MEKC experiments (Fig. 5b) and estimated as 1.4 % ± 0.1 % of the "R-isomer" content. This impurity was also observed when the mixture of FCE 23762 and "R-isomer" was investigated (see Fig. 4a). The adriamycinone sample was found to be pure with either CZE or MEKC (results not shown).

CONCLUSIONS

Since we used two different separation mechanisms for the analysis of the anthracycline analogues, the impurities in the samples could be verified very efficiently. As a validation of the methods developed in this study we did not find







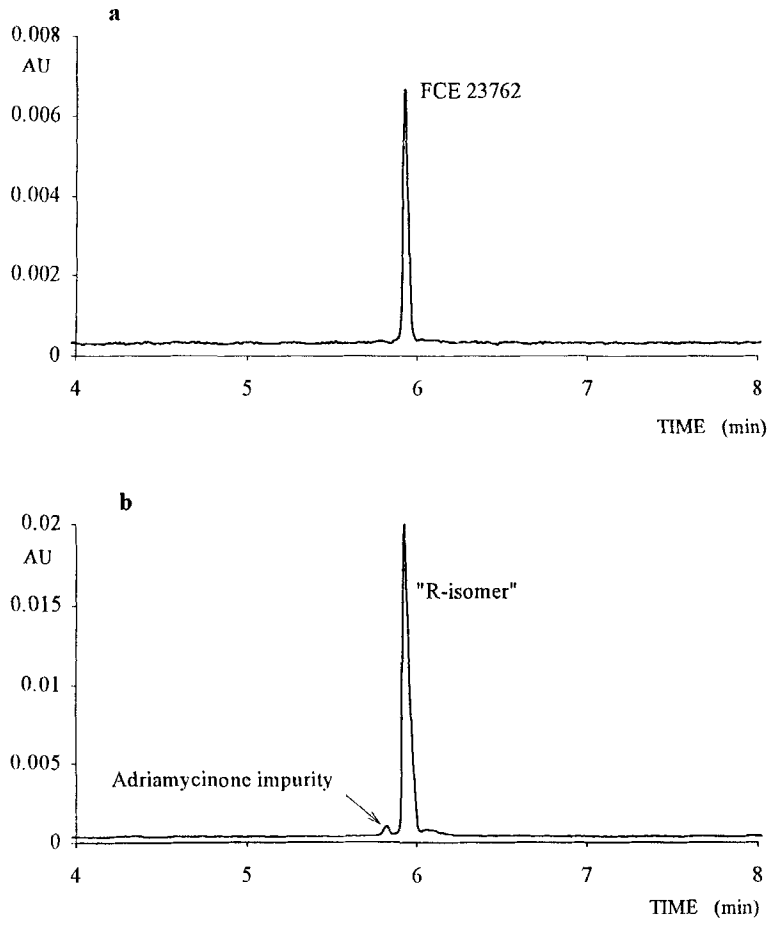


FIGURE 5. Micellar electrokinetic chromatography experiments of the (a) FCE 23762 (10 μg/ml) and (b) "R-isomer" (30 μg/ml) samples. Experimental conditions as in Fig. 4.

FIGURE 4. Micellar electrokinetic chromatography experiments of sample mixtures of (a) FCE 23762 (13 μg/ml) "R-isomer" (12 μg/ml), (b) FCE 23762 (8 μg/ml) and adriamycinone (7 μg/ml) and (c) "R-isomer" (9 μg/ml) and adriamycinone (6 μg/ml). Background electrolyte 15 mM Na-phosphate - 6 mM Na-borate, pH 7, containing 20 mM SDS. Experimental conditions: capillary 75 μm ID x 42 cm (34 cm effective length); voltage 20 kV; current 34 μA; detection 225 nm; temperature 25 °C. Sample application was made by vacuum for 1.5 s.

other impurities in the FCE 23762 sample than it was given by the producer. We were able to determine the impurities, determined by the other techniques above the detection limits of our methods. At the same time we characterized the sample of the "R-isomer" and described a small adriamycinone content in it.

The results demonstrate that the capillary electrophoresis analysis of FCE 23762, "R-isomer" and adriamycinone can be performed within short run time, and with high sensitivity. This makes the technique a potential complementary one to HPLC.

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