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Stability studies with a high-performance liquid chromatographic method for the determination of a new anthracycline analogue, 3′-deamino-3′-[2-(*S*)-methoxy-4-morpholino]doxorubicin (FCE 23762), in the final drug formulation

Maria Luisa Rossini and Marina Farina*

Galenical Research and Development, Farmitalia Carlo Erba, Via Carlo Imbonati 24, 20159 Milan (Italy)

ABSTRACT

A high-performance liquid chromatographic method was studied to optimize the separation of FCE 23762, a new antitumour agent, from both synthetic impurities and degradation products having very similar molecular structures. The main problems faced in the analytical method development using the most common reversed-phase columns available arose from the presence of analytical peaks with poor symmetry, a long analysis time and the separation between FCE 23762 and its *R*-isomer, which was often unsuitable for the correct determination of the drug substance. The use of a new stationary phase, Zorbax R_x-C8, together with a suitable mobile phase resulted in a good separation between the diastereomers, with satisfactory peak symmetry and run time. The method permitted the study of the stability of the drug substance in formulations for clinical trials.

INTRODUCTION

3′-Deamino-3′-[2-(*S*)-methoxy-4-morpholino]-doxorubicin (Fig. 1), laboratory code FCE 23762, is a new antitumour agent belonging to the anthracycline group, showing marked growth-inhibiting properties similar to those of its parent drug, doxorubicin [1], whose clinical use in acute leukaemias, malignant lymphomas and solid tumour is well known [2–7].

In order to obtain good separation and selectivity among anthracyclines, different analytical methods [8–26] have been developed in the last decade, both for stability and pharmacokinetic studies, applying a wide variety of reversed-phase columns. The application of the official analytical method for doxorubicin [27], which involves the use of a reversed-phase trimethylsilane column (Zorbax TMS), was unsatisfactory, mainly owing to the low level of peak symmetry achieved. The use of a new stationary phase with deactivated silanol groups [28,29], Zorbax R_x-C8, designed for the analysis of basic and polar compounds, and optimization of the analytical conditions (mobile phase pH, buffer concentration and temperature) allowed us to develop an appropriate method for the determination of the active drug and for its separation from related substances, especially its *R*-isomer, to be used in stability studies on final dosage forms.

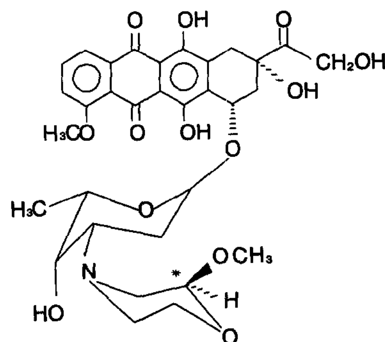

Fig. 1. Structural formula of FCE 23762.

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EXPERIMENTAL AND RESULTS

Apparatus and chromatographic conditions

The experiments were carried out on a Milton Roy (Rochester, NY, USA) CM 4000 liquid chromatograph equipped with a Zorbax R_x-C8 analytical column (25 cm × 4.6 mm I.D.; average particle size 5 μm) manufactured by Rockland Technologies (Newport, DE, USA), a Gilson (Worthington, OH, USA) Model 231 autosampler, a Shimadzu (Tokyo, Japan) SPD-6A spectrophotometric detector and a Spectra-Physics (San Jose, CA, USA) Model SP 4270 integrating recorder.

The analytical wavelength chosen was 254 ± 1 nm. The mobile phase was water–acetonitrile (70:30, v/v), containing 2 ml/l of 85% H_3PO_4, adjusted to pH 6.0 with 2 *M* NaOH. A LABNET/IBM system (Spectra-Physics) was used for collecting and processing data.

Sample solutions were stored at ambient temperature and used within 24 h of preparation.

Chemicals

FCE 23762, its *R*-isomer, the synthetic intermediate doxorubicin and the most probable degradation product likely to be present, adriamycinone, were kindly supplied by Carlo Erba R&D Chemical Department. The chemicals used were of analytical-reagent grade and the solvents for the high-performance liquid chromatographic (HPLC) analyses were of HPLC grade.

Mobile phase study

Water to acetonitrile ratio. During the trials on mobile phase optimization, a solution in water–acetonitrile (50:50, v/v) of FCE 23762 (about 50 μg/ml), the *R* diastereomer (about 15 μg/ml) and adriamycinone (about 10 μg/ml) was injected into the chromatographic system and analysed using mobile phases with different proportions of the two solvents.

After several experiments with mobile phase compositions varying from 50:50 to 90:10 (v/v), the optimum composition was found to be water–acetonitrile (70:30, v/v), which gave the best results for the determination of related substances with low-polarity characteristics.

Influence of pH. Water–acetonitrile (70:30, v/v) mixtures were prepared and adjusted to different pH values in the range 2.5–7.5 by means of 85% H_3PO_4 and 2 *M* NaOH. The ionic strength was kept constant at 0.08 by adding NaCl in appropriate

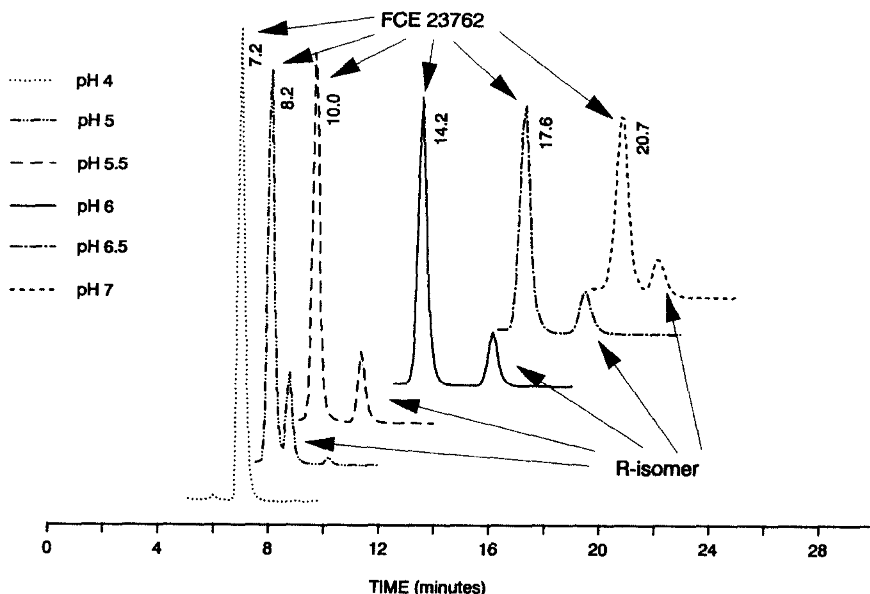

Fig. 2. Chromatograms showing the separation of FCE 23762 from its *R*-diastereomer when the pH of the mobile phase is varied (I = 0.08).

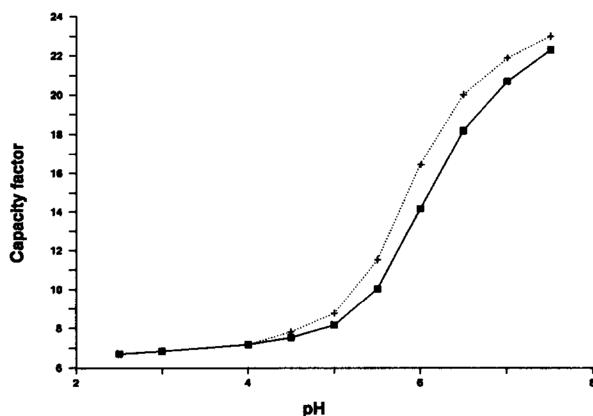

Fig. 3. Capacity factors (k') of (■) FCE 23762 and (+) its *R*-isomer *versus* pH.

amounts to guarantee that the variations observed in the chromatographic traces were dependent only on the pH effect.

The chromatograms obtained (Fig. 2) show that the retention times of both diastereomers increase as the pH increases, and the optimum symmetry of the peaks is achieved at low pH values.

Plots of the capacity factor (k') *versus* pH are given in Fig. 3 for FCE 23762 and its *R*-isomer and indicate that only the pH range *ca.* 4.5–7.5 can be considered suitable for the separation of the two isomers. Complete separation of the diastereomers is obtained solely in the pH range 5.5–6.5 as shown by the resolution values [30] reported in Table I, which also indicates that pH 6.0 is the optimum for resolution of the peaks.

As the k' values of the two ionizable solutes in phases buffered at different pH values depend on their pK_a values [31–33] and the major variations of this parameter are observed at pH values close to the pK_a value, it is likely that the separation between the two diastereomers under our analytical conditions is strongly influenced by their different pK_a values (6.25 and 6.02 for FCE 23762 and its *R*-isomer, respectively, determined by potentiometric titration).

TABLE I

RESOLUTION BETWEEN THE PEAKS OF FCE 23762 AND ITS *R*-ISOMER *VERSUS* pH

pH	Resolution	pH	Resolution
4.0	0	6.0	4.2
5.0	1.2	6.5	3.2
5.5	2.3	7.0	1.6

Influence of buffer concentration. To the water–acetonitrile (70:30, v/v) mobile phase were added phosphate buffer (pH 6.0) at different concentrations (from 0.0008 to 0.4 *M*), and the resulting mixtures were tested for the separation of FCE 23762 and its *R*-isomer. On increasing the buffer concentration the symmetry of the peaks improves and the retention times of both the isomers decrease; on lowering the buffer concentration the elution time of the two isomers is reversed.

Optimum separation of the isomers and peak symmetry were obtained when 2 ml/l of 85% H_3PO_4 were added to the mobile phase, the pH then being adjusted to 6.0 with 2 *M* NaOH (buffer concentration = 0.04 *M*). Under these conditions, the separation of related substances from the main peaks was also better, allowing a more precise determination.

Influence of temperature. The influence of temperature on the separation of FCE 23762 from its *R*-isomer was studied using the optimized mobile phase on a Zorbax R_x-C8 column maintained at 2, 12, 22, 32, 42 and 52°C by means of a thermostat.

At higher temperatures (32, 42 and 52°C) the symmetry of the peaks decreases and the retention times increase, and at the lowest (2°C) and highest (52°C) temperatures tested the separation of the *S*- and *R*-isomers is significantly reduced, hence the optimum column temperature is 22°C. Therefore, for practical reasons, the stability studies on FCE 23762 formulations can be conducted at room temperature.

Validation of the HPLC method

The method was validated for the assay of freeze-dried vials dosed at 50 and 500 μg, and showed the following performances.

Precision. Repeated determinations on six different samples showed a relative standard deviation (R.S.D.) of 0.73% for the 50-μg dosage and R.S.D. = 0.56% for the 500-μg dosage.

Accuracy. Samples prepared extemporaneously with FCE 23762 in amounts close to the limits of acceptance (85–115% of the label claim) showed an accuracy of 99.55 ± 0.75% for the 50-μg dosage and

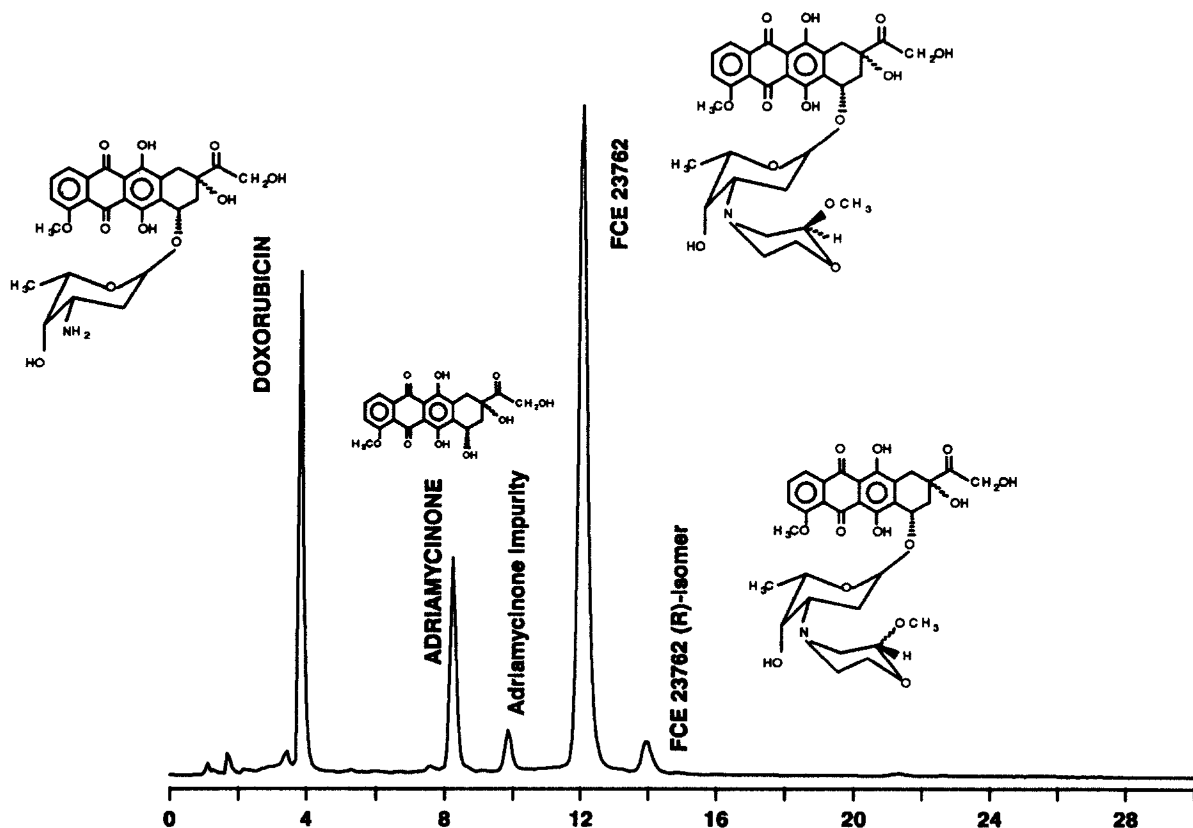

Fig. 4. Chromatogram of a mixture of FCE 23762, its *R*-diastereomer, doxorubicin and adriamycinone analysed under the optimized experimental conditions.

99.67 ± 0.88% for the 500-μg dosage (both as means of twelve determinations).

Linearity. Calibration standard solutions prepared spanning a concentration range of 10–160 μg/ml showed a correlation coefficient $r = 0.999687$.

Specificity. The method is capable of assaying FCE 23762 in the final dosage forms without interference arising from the presence of structurally related compounds. This was confirmed by chromatographing a mixture of FCE 23762 (*ca*. 50 μg/ml), its *R*-isomer (*ca*. 5 μg/ml), doxorubicin (*ca*. 30 μg/ml) and adriamycinone (*ca*. 15 μg/ml) dissolved in water–acetonitrile (70:30, v/v) under the optimized analytical conditions (Fig. 4).

Stability-indicating power. The method allows the determination of FCE 23762 in samples forcibly degraded under acidic and basic conditions, under intense white light and in the presence of a strong oxidizing agent, without interference arising from the side-products formed (Fig. 5 and Table II).

Stability trials

The HPLC method was applied to the assay of FCE 23762 in its final dosage forms, freeze-dried vials dosed at 50 and 500 μg, with the aim of determining the provisional shelf-life of the drug product for clinical studies. The freeze-dried vials, supplied by Carlo Erba Galenical Development Department, were reconstituted with water–acetonitrile (70:30, v/v) at concentrations of 50 and 100 μg/ml, respectively, and then injected into the chromatographic system. Tables III and IV show the stability results obtained for FCE 23762, its *R*-isomer and total related substances.

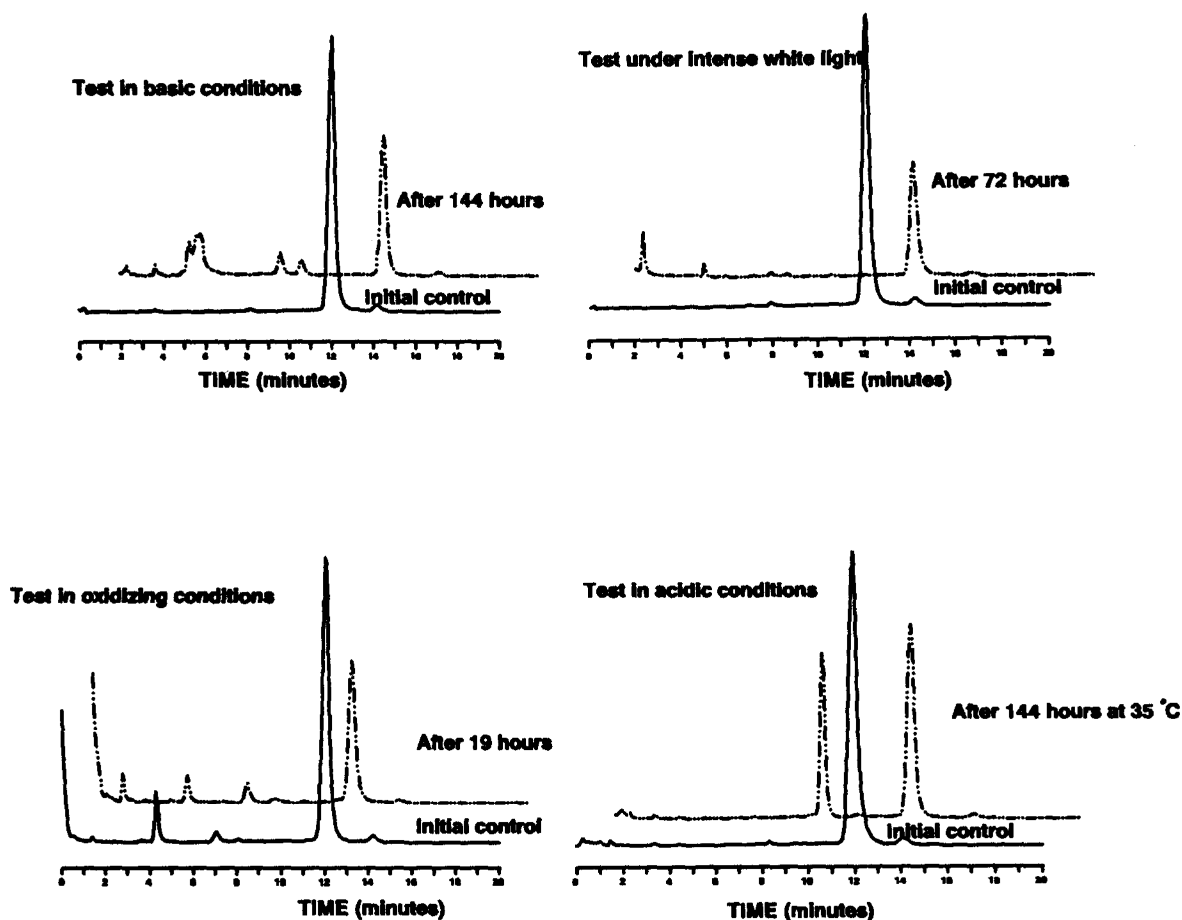

Fig. 5. Stability-indicating power of the HPLC method: chromatograms showing the forced degradation of FCE 23762 under acidic and basic conditions, in the presence of a strong oxidizing agent and under intense white light.

TABLE II

STABILITY-INDICATING NATURE OF THE HPLC ASSAY METHOD FOR FCE 23762

Results relative to initial control = 100%.

Conditions	Time (h)	Residual concentration (%)
Acidic	144	53.8
Basic	144	48.2
Intense white light	72	39.9
Oxidizing	19	52.1

CONCLUSIONS

The results demonstrate the excellent power of the Zorbax R_x-C8 column in reducing peak tailing and giving contant performance and high efficiency for long periods. The developed and validated HPLC method proved capable of identifying and separating FCE 23762 from related substances and in particular from its *R*-diastereomer, and can be applied to the assay of the active principle in its final dosage forms to establish the shelf-life of the drug product.

TABLE III

STABILITY DATA FOR FCE 23762

50-μg freeze-dried vials, batch No. TF/23659. R.S. = Related substances.

Temperature (°C)	Parameter	Initial control	1 month	2 months	3 months
55	Assay (%)	100.0	102.2		
	Total R.S. (%)	3.33	3.74		
	R-isomer (%)	1.89	2.35		
45	Assay (%)	100.0	100.9	100.7	96.8
	Total R.S. (%)	3.33	3.80	3.68	3.47
	R-isomer (%)	1.89	2.44	2.52	2.09
35	Assay (%)	100.0	102.3	102.2	100.1
	Total R.S. (%)	3.33	3.54	3.28	3.10
	R-isomer (%)	1.89	2.36	2.19	1.99
30	Assay (%)	100.0	102.9	102.1	98.2
	Total R.S. (%)	3.33	3.69	3.69	3.15
	R-isomer (%)	1.89	2.40	2.29	2.07
25	Assay (%)	100.0	102.3	101.1	101.4
	Total R.S. (%)	3.33	3.25	3.60	3.41
	R-isomer (%)	1.89	2.27	2.33	2.11

TABLE IV

STABILITY DATA FOR FCE 23762

500-μg freeze-dried vials, batch No. TF/23660.

Temperature (°C)	Parameter	Initial control	1 month	2 months	3 months
55	Assay (%)	100.0	101.6		
	Total R.S. (%)	3.47	3.92		
	R-isomer (%)	2.20	2.48		
45	Assay (%)	100.0	102.3	99.9	98.7
	Total R.S. (%)	3.47	3.83	3.50	3.58
	R-isomer (%)	2.20	2.44	2.26	2.09
35	Assay (%)	100.0	101.0	100.5	98.2
	Total R.S. (%)	3.47	3.77	3.66	3.51
	R-isomer (%)	2.20	2.47	2.22	2.14
30	Assay (%)	100.0	100.5	99.9	99.1
	Total R.S. (%)	3.47	3.75	3.77	3.45
	R-isomer (%)	2.20	2.47	2.18	2.17
25	Assay (%)	100.0	101.7	101.0	98.8
	Total R.S. (%)	3.47	3.73	3.63	3.37
	R-isomer (%)	2.20	2.47	2.28	2.19

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