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Short communication

A HPLC method for the simultaneous determination of seven anthracyclines

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Abstract

A HPLC method has been developed for the determination of epirubicin hydrochloride, doxorubicin hydrochloride and idarubicin hydrochloride in the presence of four other anthracyclines. This method ensures the rapid determination of seven anthracyclines. It is simple and rapid and does not require any preliminary treatment of the sample. The method was fully validated.

Keywords: Anthracyclines; Epirubicin hydrochloride; Doxorubicin hydrochloride; Idarubicin hydrochloride; HPLC

1. Introduction

Anthracyclines were originally isolated from a pigment-producing *Streptomyces* and are among the most widely used anticancer agents. From the 1960s to the present day, more than 200 anthracyclines, which occur naturally, have been identified and hundreds of derivatives have been synthesized in order to overcome their high toxicity and multi-drug resistance. To the best of our knowledge, the most frequently used of anthracyclines is at present epirubicin hydrochloride [1,2]. A judicious scrutiny of scientific literature has revealed the fact that epirubicin hydrochloride is a drug with a more favourable therapeutic index than doxorubicin hydrochloride [3]. At present, there is only one official monograph in pharmacopoeia for the quality-control of epirubicin hydrochloride [4] even if many scientific papers have reported chromatographic methods like HPLC with DAD [5–8], electrochemical or fluorescence detection [9,10] and, more recently, LC–MS methods [11–13].

It is for this reason that a rapid, accurate, selective and sensitive quality-control method for anthracycline raw materials appears to be extremely valuable for the pharmaceutical industry.

This paper reports a HPLC method with DAD detection that has been developed for the simultaneous determination of seven anthracyclines: on one hand, epirubicin hydrochloride, doxorubicin hydrochloride, idarubicin hydrochloride as major compounds, and on the other hand, doxorubicinone, daunorubicinone, daunorubicin and epi-daunorubicin as related substances. The method was fully validated according to the ICH rules [14].

2. Experimental

2.1. Materials

The study was conducted with seven standards from the European Pharmacopoeia (Strasbourg, France): epirubicin hydrochloride; doxorubicin hydrochloride; idarubicin hydrochloride; doxorubicinone; daunorubicinone; daunorubicin; epi-daunorubicin. The water used for chromatography was purified by means of a MilliQ ultra-pure water-system Biocel A 10, Millipore (Yvelines, France) and the acetonitrile gradient grade was purchased from Aldrich (Taufkirchen,

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Germany). The phosphoric acid 85% ($\rho = 1.71\ g\ mL^{-1}$) was purchased from Riedel de Haën (Seelze, Germany) and sodium dodecylsulfate (Ph. Eur., DAB, BP Pharm.) from Merck (Darmstadt, Germany).

2.2. Equipment

Two Hewlett-Packard 1100 chromatographic systems (Boeblingen, Germany) were employed. System 1 consisted of a quaternary pump G 1311A, a PDA detector G 1315A, an automatic injector G 1313A, a column thermostat G 1316A and an on-line degasser G 1322A. The chromatographic system 2 consisted of a binary pump G 1312A, a PDA detector G 1315A, an automatic injector G 1313A, a column thermostat G 1316A and an on-line degasser G 1322A. The chromatographic separations were carried out using a column purchased from Agilent Technologies (Waldbroun, Germany) type Zorbax Eclipse XDB–C8, 5 μm, 25 cm × 4.6 mm with column temperature 35 °C. The detection was at $\lambda = 254 \pm 2$ nm, reference $\lambda = 360 \pm 2$ nm. The data were acquired and processed by means of HP ChemStation for LC software. The ORIGIN program (Micro Cal Inc., Version 4.10.) was employed for the linear regression analysis.

2.3. Separation studies

Separations were achieved with a mobile phase formed by mixing the solvent A (aqueous solution with pH 2.00, containing 0.4% sodium dodecylsulfate) with the solvent B (mixture of methanol:acetonitrile = 1:1, v/v). The composition of the mobile phase was: solvent A:solvent B = 40/60. The injection volume was 20 μL, and the mobile phase flow rate was kept constant at 2 mL min^{-1}.

2.4. Stock solutions

All the samples were prepared by dissolution in the mobile phase and were filtered by a 0.45-μm filter. The concentration of the stock solution for epirubicin hydrochloride, doxorubicin hydrochloride and idarubicin hydrochloride, respectively, was 1.00 mg mL^{-1} and the solutions were prepared by dissolving an appropriate quantity of each compound in the mobile phase. The concentration of each of the stock solutions of doxorubicinone, daunorubicinone, daunorubicin and epi-daunorubicin was 0.50 mg mL^{-1}.

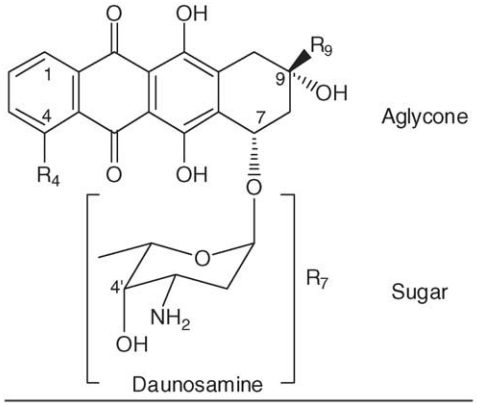
2.5. Validation of the method

2.5.1. Linearity and range

Aliquots of the stock solutions of each compound were placed in 10 mL volumetric flasks, which were filled up with water. For the calibration graph, successive dilutions were performed using 10 or 5 mL volumetric flasks.

2.5.2. Precision

Six different concentrations of each compound were analysed on the same day and the values of R.S.D. were calculated to determine the intra-day precision. The same procedure was performed on different days; thus, the inter-day precision was determined, too.



No*.	Anthracycline	R_4	R_9	R_7
1	Doxorubicinone	$-OCH_3$	$-C(=O)CH_2OH$	–OH
2	Daunorubicinone	$-OCH_3$	$-C(=O)CH_3$	–OH
3	Doxorubicin	$-OCH_3$	$-C(=O)CH_2OH$	Daunosamine
4	Epirubicin	$-OCH_3$	$-C(=O)CH_2OH$	Daunosamine (4' epimer)
5	Daunorubicin	$-OCH_3$	$-C(=O)CH_3$	Daunosamine
6	Epi-Daunorubicin	$-OCH_3$	$-C(=O)CH_3$	Daunosamine
7	Idarubicin	–H	$-C(=O)CH_3$	Daunosamine

*The number of compound according to the elution order

Fig. 1. The chemical structure of anthracyclines.

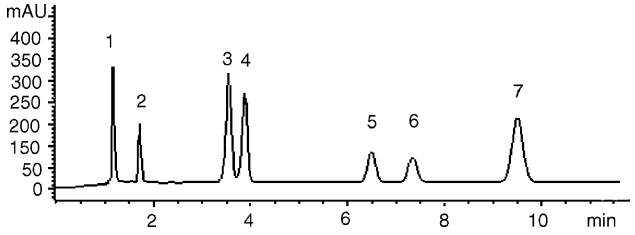


Fig. 2. Chromatogram for system suitability: solvent A/solvent B = 40/60; the injection volume, 20 μL; temperature, 35 °C; the flow rate, 2 mL min^{-1}; (**1**) doxorubicinone; (**2**) daunorubicinone; (**3**) doxorubicin; (**4**) epirubicin; (**5**) daunorubicin; (**6**) epi-daunorubicin; (**7**) idarubicin.

2.5.3. Accuracy

Accuracy was evaluated by fortifying a mixture containing epirubicin hydrochloride, doxorubicin hydrochloride and idarubicin hydrochloride with known concentrations of doxorubicinone, daunorubicinone, daunorubicin and epi-daunorubicin. The recovery of each compound was calculated.

2.5.4. Ruggedness

The ruggedness of the procedure was checked after the following parameters had been altered deliberately; the composition of the mobile phase, the temperature and the mobile phase flow rate in the following variants: 1.8 and 2.2 mL min^{-1}. These variants represent ±10% of the proposed flow rate (2 mL min^{-1}).

3. Results and discussion

The chemical structure of the epirubicin hydrochloride, doxorubicin hydrochloride, idarubicin hydrochloride, which it is possible to find as a major compound in pharmaceutical forms, is shown in Fig. 1. The composition of the mobile phase was adjusted until a proper separation between those anthracyclines and doxorubicinone, daunorubicinone, daunorubicin and epi-daunorubicin was obtained. All the compounds under examination were separated rapidly and completely.

3.1. Validation of the method

3.1.1. Specificity and selectivity

The specificity/selectivity of the analytical method was confirmed by the analysis of solutions that contained 100% of the normal working concentration (0.1 mg mL^{-1}) of the epirubicin hydrochloride, doxorubicin hydrochloride and idarubicin hydrochloride and 25% of the related substances, i.e. doxorubicinone, daunorubicinone, daunorubicin and epi-daunorubicin (Fig. 2). The parameters of the separation were the same for the solution containing 100% of the normal working concentration and 0.1% of the related impurities.

Samples containing the above-mentioned seven anthracyclines and excipients for lyophilization have been analysed. The chromatograms obtained in this manner are similar to the one presented in Fig. 2. The method is selective by regard to the excipients for lyophilization.

Table 1
Results of the linearity studies

Compound	$y = A + Bx$		R^a
	A	B	
Doxorubicin	215.07	24043.92	0.9991
Epirubicin	105.48	20724.46	0.9992
Idarubicin	−289.38	42106.34	0.9994
Doxorubicinone	−0.08	13539.54	0.9985
Daunorubicinone	0.20	49158.14	0.9976
Daunorubicin	−0.15	36146.51	0.9968
Epi-daunorubicin	2.81	35874.41	0.9953

[a] R, coefficient of correlation.

3.1.2. Linearity and linear dynamic range

The linearity of the method used for each anthracycline assay was evaluated on a calibration curve of the peak area (y, mAU s) versus the concentration of the analyte (x, mg mL^{-1}). Each sample was prepared in triplicate and nine different concentrations were used for the linearity studies. The equations of linear regression obtained for various concentrations in the range of 10–120% of the normal analytical working concentration are summarized in Table 1. The intercept is very small and the correlation coefficient is close to unity. The values that have been obtained show a good linearity and the fit of Beer's law.

The detection limit was established by assessing the signal-to-noise ratio level in a proportion of 3:1 and the quantitation limit for the signal-to-noise ratio in a proportion of 10:1. The results are presented in Table 2.

3.1.3. Precision

Precision was estimated by determining repeatability and intermediate precision. Repeatability, also defined as 'intra-assay' precision, was evaluated by six consecutive measurements performed on simulated solutions at a concentration of 100% of the normal analytical working value. Intermediate precision was determined by evaluating the repeatability of the investigated method (analytical procedure), which was reproduced in the same laboratory, but under different operational conditions: with a different investigator, a different column (different batch, same manufacturer), or with the

Table 2
Determinations of LOD and LOQ for doxorubicin, epirubicin, idarubicin, doxorubicinone, daunorubicinone, daunorubicin and epi-daunorubicin

Compound	LOD (ng mL^{-1})	LOQ (ng mL^{-1})
Doxorubicin	110	38
Epirubicin	128	45
Idarubicin	162	55
Doxorubicinone	36	144
Daunorubicinone	29	75
Daunorubicin	42	138
Epi-daunorubicin	75	250

Table 3
Results of the precision and accuracy studies for doxorubicin, epirubicin and idarubicin

	Doxorubicin	Epirubicin	Idarubicin
Precision (% ± S.D.) repeatability	100.0 ± 0.10	99.3 ± 1.03	99.6 ± 1.32
Intermediate precision			
Day I	99.0 ± 0.39	99.8 ± 0.45	100.2 ± 1.40
Day II	100.3 ± 1.20	98.8 ± 1.56	99.7 ± 1.80
Accuracy (% ± S.D.)	98.9 ± 0.68	99.8 ± 1.25	100.0 ± 1.62

analysis carried out on another day. The precision of the method had a relative standard deviation (R.S.D.) below 2%, which complies with the acceptance criteria proposed (R.S.D.: not more than 2.0%). The results are presented in Tables 3 and 4.

3.1.4. Accuracy

Accuracy was determined on the range of 80–120% of the analytical working concentration of each compound by means of calculating the recovery. Mixtures of the studied anthracyclines – corresponding to three concentration levels namely 80, 100 and 120% of analytical working concentration – were analyzed. Each concentration level was prepared for three times.

Method accuracy, determined in the interval 80–120% of the working concentration and evaluated by the parameter "recovery" was within the proposed limits (100 ± 2%). The results are presented in Tables 3 and 4.

3.1.5. Ruggedness

The ruggedness of the procedure was checked after the following parameters had been altered deliberately: the composition of the mobile phase, the mobile phase flow rate in the variants: 1.8 and 2.2 mL min^{-1}, which represent ±10% of the proposed flow (2 mL min^{-1}) and the temperature in the range 33–37 °C (±2 °C). For each case, the influence of changes on the performance of the chromatographic system in the area of interest and on the retention time was evaluated. No important changes in resolution (±0.15) and area (±2%) were observed, when modifications in the temperature and flow rate were studied. The variation of the composition of the mobile phases determines a dramatic change in the whole time of the analysis (Tables 5 and 6). Method ruggedness, checked after deliberate alterations of the mobile-phase composition, flow rate and temperature shows that the changes of the operational parameters do not lead to essential changes of the performance of the chromatographic system (resolution, retention time for the peak of interest), but to an increase of the time of analysis, when the composition of the mobile phase has been changed.

3.1.6. Stability of the solution

The chemical stability of anthracyclines in the stock solution was studied for a period of 12 days. The solutions were kept both at 2–8 °C and at room temperature. The solutions studied were stable in a solution stored for 12 days at 2–8 °C and in a solution stored for 5 days at room temperature. The retention time of each compound was in the range: initial retention time ± 0.5 min, and the area of each peak was in the range: initial area ± 2%. No other peaks were observed during the stability studies.

3.1.7. Applications

The method has been applied to pharmaceutical formulations containing epirubicin hydrochloride, doxorubicin hydrochloride, idarubicin hydrochloride as major compounds, doxorubicinone, daunorubicinone, daunorubicin and epi-daunorubicin as related substances and excipients for lyophilization. The recoveries were between 99.5 and 101.2% for the major compounds and between 98.6 and 101.9% for related substances. These values are in agreement with those obtained, when the official monograph was applied

Table 4
Results of the precision and accuracy studies for doxorubicinone, daunorubicinone, daunorubicin and epi-daunorubicin

	Doxorubicinone	Daunorubicinone	Daunorubicin	Epi-daunorubicin
Precision (% ± S.D.) repeatability	99.8 ± 0.26	99.1 ± 1.09	99.4 ± 0.67	100.0 ± 1.08
Intermediate precision				
Day I	98.9 ± 1.64	100.1 ± 1.51	99.5 ± 1.80	99.3 ± 1.72
Day II	99.2 ± 0.45	99.5 ± 1.18	98.7 ± 1.09	99.6 ± 0.50
Accuracy (% ± S.D.)	98.6 ± 0.59	99.4 ± 1.53	98.1 ± 1.65	99.0 ± 1.24

Table 5
Results of robustness studies for doxorubicin, epirubicin and idarubicin

Solvent A/solvent B	t_R ± S.D. (min)		
	Doxorubicin	Epirubicin	Idarubicin
40/60	3.55 ± 0.15	3.87 ± 0.85	9.50 ± 0.21
50/50	6.58 ± 0.69	11.44 ± 0.65	31.18 ± 0.28
60/40	13.09 ± 0.98	18.84 ± 0.43	48.19 ± 0.34

Table 6
Results of robustness studies for doxorubicinone, daunorubicinone, daunorubicin and epi-daunorubicin

Solvent A/solvent B	$t_R \pm$ S.D. (min)			
	Doxorubicinone	Daunorubicinone	Daunorubicin	Epi-daunorubicin
40/60	1.17 ± 0.20	1.70 ± 0.57	6.49 ± 0.08	7.34 ± 0.43
50/50	2.43 ± 0.15	4.56 ± 0.31	22.68 ± 0.48	27.04 ± 0.37
60/40	5.36 ± 0.23	9.25 ± 0.84	26.03 ± 0.24	32.59 ± 0.22

4. Conclusion

The suitability of the HPLC method for the determination of seven anthracyclines has been studied. The HPLC method has adequate selectivity, good linearity, sensitivity, precision, accuracy and ruggedness.

The validation report confirms the fact that the proposed HPLC method can be used as a method for the determination of such antracyclines during formulation or in finished products.

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