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DEVELOPMENT OF A REVERSED-PHASE ION-PAIRING LIQUID CHROMATOGRAPHIC ASSAY OF DAUNORUBICIN FOR PHARMACOPOEIAL USE

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SUMMARY

A liquid chromatographic method is described for the determination of daunorubicin using octyldecylsilyl columns and sodium dodecyl sulphate as ion-pairing agent. The method based on a previous publication [M. A. van Lancker *et. al., J. Chromatogr.*, 254 (1983) 45] is specific for daunoribicin and separates it from likely impurities, *e.g.*, the aglycone, duborimycin and related anthracyclines, doxorubicin and epirubicin. Several commercial batches have been examined in a number of laboratories employing various types of stationary phases. The intralaboratory reproducibilities varied from 1.0 to 1.6% and the interlaboratory reproducibility between 0.63 and 1.0% (based on the means of duplicate determinations), while with the preferred mobile phase the coefficient of variation was 0.3% ($n=4$). The method is robust and is proposed for inclusion in a monograph on daunorubicin which is currently under consideration by the European Pharmacopoeia.

INTRODUCTION

Anthracyclines, particularly daunorubicin and doxorubicin, are extensively used for treatment of cancer[2]. The drugs are now well established and as such it is desirable that their quality be assured throughout the Western European countries by common standards acceptable to all participating countries of the European Pharmacopoeia Convention[3].

During the preparation of a monograph on daunorubicin it was considered desirable to employ a specific method for the analysis of this compound which should be free from likely interference from related substances which may be byproducts of the method of manufacture, should be unaffected by the appearance of degradation products likely to be encountered during the shelf-life of the substance and should differentiate from other commercially available anthracyclines. It is particularly im-

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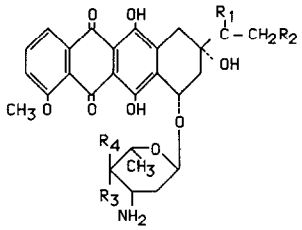

Fig. 1. Structures of daunorubicin and some related anthracyclines.

	R_1	R_2	R_3	R_4
Daunorubicin	=O	H	OH	H
Doxorubicin	=O	OH	OH	H
Duborimycin	H(OH)	H	OH	H
Epirubicin	=O	OH	H	OH

portant that the method should be capable of determining the extent of degradation to the aglycone since this substance has little antitumour activity and is particularly toxic. The structure of the anthracyclines of interest are shown in Fig. 1.

Since the ultraviolet–visible spectra and the fluorescence yields are similar for the anthracyclines and their aglycones, due to the common dehydroxyanthraquinone chromophore[5], liquid chromatography was considered as the most likely approach. Normal- and reversed-phase silica with C_{18}[4–6], C_8[7] or phenyl bonded[8] systems have been proposed, sometimes with a gradient elution[8] system, for the separation of glycosylated roducts from the aglycones. The many methods employed have been reviewed[9].

In a pharmacopoeial test it was thought inappropriate to employ a gradient elution technique which could have problems of reproducibility in the various laboratories obliged to employ such a method. Thus both normal-phase silica and reversed-phase isocratic systems have been investigated and the results are presented in this paper.

EXPERIMENTAL

Chemicals

All solvents employed were of analytical grade (E. Merck, Darmstadt, F.R.G.; Prolabo, Rhône-Poulenc, France). Sodium dodecyl sulphate was obtained from Fluka (Buchs, Switzerland). Daunorubicin, duborimycin and daunorubicin aglycone were supplied by Rhône-Poulenc (Vitry sur Seine, France), doxorubicin and epirubicin by Farmitalia (Milan, Italy).

Apparatus

The liquid chromatographic system consisted of a Knauer double piston pump with a Hewlett-Packard 1040A multi-diode array detector linked to an HP858 microcomputer and an HP3390A integrator, or a Chromatem 380 pump with a spectromonitor LDC III linked to a Shimadzu C-R3 A integrator.

TABLE I

VARIOUS CHROMATOGRAPHIC CONDITIONS EMPLOYED USING SILICA AS STATIONARY PHASE

The solvent was methanol–water–formic acid buffer pH 2.5–dichloromethane (130:6.5:0.75:863) and the wavelength of detection was 254 nm.

System	*Column dimensions (mm)*	*Stationary phase*	*Flow-rate (ml/min)*
A	250 × 4.6	LiChrosorb Si 60 (5 μm)	1.5
B	250 × 4.6	LiChrosorb Si 60 (5 μm)	3.0
C	100 × 4.6	LiChrosorb Si 60 (5 μm)	1.0
D	100 × 4.6	Nucleosil 50 5 μm	1.0
E	100 × 4.6	Spherisorb 5 μm	1.0

Preliminary experiments

(a) Various normal-phase silica supports were employed (Table I) using as mobile phase: methanol–water–formic acid buffer pH 2.5–dichloromethane (130:6.5:0.75:863)[10]. The formic acid buffer was prepared by mixing 30 ml water, 18 ml formic acid, 10 g ammonium chloride and 9 g ammonium hydroxide and diluting to 100 ml in water. Solutions of the anthracyclines (%, m/v) were prepared in the mobile phase as follows: daunorubicin, 0.05; daunorubicin aglycone, 0.01; duborimycin, 0.01; doxorubicin, 0.01.

(b) A 250 mm × 4.6 mm stainless-steel column was slurry packed in the laboratory with reversed-phase silica C_{18}, 5 μm. The mobile phase was 0.01 *M* sodium dodecyl sulphate, 0.02 *M* phosphoric acid in acetonitrile–water (50:50). The types of stationary phase employed were Lichrosorb RP-18 (5 μm), Spherisorb C_{18}, Nucleosil 5 C_{18}, Partisil C_{18} ODS-3, Hypersil ODS 5 μm and μBondapak C_{18} (10 μm).

For the calculation of theoretical plates and the resolution factor, the methods described in the General Prescriptions of the European Pharmacopoeia were followed (V.6.2.1)[18].

Alteration of the concentration of the organic modifier was investigated (first collaborative experiment). Chromatographic column: 250 × 4.6 mm chemically octadecyl bonded silica. Mobile phase: acetonitrile–water mixture (50:50), containing 0.01 *M* sodium dodecyl sulphate and 0.02 *M* phosphoric acid. Detection wavelength: 254 nm. The content of the organic modifier may need slight adjustment so as to obtain the desired separation.

Suitability test

Into a tared 10-ml volumetric flask were weighed 10 mg of daunorubicin and 10 mg duborimycin. These were dissolved in the mobile phase and the solution diluted to volume in the same solvent. A 20-μl volume of this solution was injected via a loop injection device and the operating were adjusted so that the peaks obtained were within 70–90% full scale deflection. After another injection of the suitability test solution, the retention times of the reference compounds were recorded. The relative retention times of the components of the reference mixture are as follows: daunorubicine, 1.00; duborimycine 0.68.

For each compound the retention times of the two replicate injections must be within 3.5%. The retention time of daunorubicin is about 20 min. The number of theoretical plates, N, was calculated at a suitable chart speed. For the peak of daunorubicin, N is 10000 ± 1000. For the same chromatogram, the resolution, R_s, between daunorubicin and duborimycin was calculated (4.0). The symmetry factor of the daunorubicin peak (0.8–1.2) was also calculated.

Assay

The operating parameters were adjusted so that the peak obtained with the reference solution was 70–90% of full scale deflection. The use of an electronic integrator is advisable. The *test solution* was prepared by dissolving 50.0 mg of the sample in the mobile phase and diluting to 50 ml in the same solvent. A *reference solution* was prepared by dissolving 50.0 mg of daunorubicin standard in the mobile phase and diluting to 50.0 ml in the same solvent.

The second collaborative experiment was carried out in the same way as the first collaborative experiment except that the mobile phase was acetonitrile–methanol–water (45:5:50) containing 0.01 M sodium dodecyl sulphate and 0.02 M phosphoric acid.

RESULTS AND DISCUSSION

Various types of silica columns were examined (Table I) to establish whether there were any important differences in their performances. The capacity factors, k', the separation coefficient between each ingredient and the symmetry factor of the daunorubicin peak (Table II) were calculated. From the results it would appear that system D (Nucleosil 50 5 μm) (Fig. 2) is superior, giving good separation coefficient (>1.0) and a good symmetry factor (0.8–1.2), but there was quite a difference between the phases examined. Two typical batches of daunorubicin as supplied were examined using system B–E. From the results in Table III it is seen that system D separated five impurities two of which were unknown, whereas the other systems were unable to separate the same number of impurities. Apart from the aglycone and duborimycin, three other impurities exist which have the same retention time as doxorubicin. It was also noted that one impurity remained unresolved from the principal peak.

TABLE II

CALCULATED CAPACITY FACTORS, k', SEPARATION COEFFICIENTS, α, AND SYMMETRY FACTORS USING THE DIFFERENT CHROMATOGRAPHIC SYSTEMS

Chromatographic system	*Daunorubicin, k'*	*Doxorubicin*		*Duborimycin*		*Symmetry factor*
		α	k'	α	k'	
B	3.14	1.65	5.17	1.28	6.63	0.7
C	3.27	1.79	5.85	1.28	7.51	0.75
D	4.49	1.88	8.46	1.21	10.29	0.86
E	3.96	1.83	7.25	1.25	9.04	0.61

TABLE III

ANALYSIS OF TWO BATCHES OF DAUNORUBICIN (107, 114) USING DIFFERENT CHROMATOGRAPHIC CONDITIONS AND A PEAK AREA "NORMALIZATION" TECHNIQUE

ND = Not detected; SMP = shoulder on main peak, not integrated; DI = detected but not integrated. Unknown c has the same retention time as doxorubicin. Values in %.

Substance	*107*				*114*			
	Chromatographic system				*Chromatographic system*			
	B	*C*	*D*	*E*	*B*	*C*	*D*	*E*
Aglycone daunorubicin	0.41	0.46	0.39	0.41	1.68	1.56	1.56	1.56
Unknown a	0.68	0.87	1.13	0.65	0.82	1.06	1.24	0.75
Daunorubicin	98.81	98.00	96.93	98.73	97.50	96.61	95.96	97.51
Unknown b	ND	SMP	0.18	ND	ND	SMP	0.22	ND
Unknown c	ND	0.06	0.34	ND	ND	0.14	0.26	ND
Duborimycine	0.11	0.26	0.36	0.14	DI	0.24	0.33	0.11

Thus based on this evidence, there seems to be considerable variation in the silicas employed, certainly with regard to the anthracyclines, and consequently it was considered that the use of normal-phase chromatography was unsuitable as a pharmacopoeial method of control since such a method must be transferable from one laboratory to another. It was also noted that on consecutive injections of samples there appeared to be an alteration in the retention times of the more strongly retained anthracyclines (Table IV) which is not surprising considering that the mobile phase contains water. This is another disadvantage of this approach since long equilibration times could be required before any analytical procedure could be carried out.

It has previously been observed[11] when performing high-performance liquid chromatography of anthracyclines that more reproducible retention times and less tailing of the peaks were achieved with reversed-phase columns. A recent publication[12] described reversed-phase ion-pairing systems for the separation of anthracyclines. The system considered as optimal employed bonded octyldecylsilane while the mobile phase consisted of a mixture of acetonitrile and water containing sodium

TABLE IV

RESULTS FROM CONSECUTIVE INJECTIONS OF A TEST MIXTURE OF ANTHRACYCLINES USING CHROMATOGRAPHIC SYSTEM D

Experiment No.	*Retention time (min)*			
	Aglycone	*Daunorubicin*	*Doxorubicin*	*Duborimycin*
138	1.33	7.17	12.24	14.82
139	1.34	7.41	12.48	14.96
140	1.33	7.41	12.55	15.00
141	1.33	7.44	12.60	15.06
142	1.34	7.36	12.67	15.14
143	1.32	7.36	12.97	–
144	1.32	7.39	13.02	–

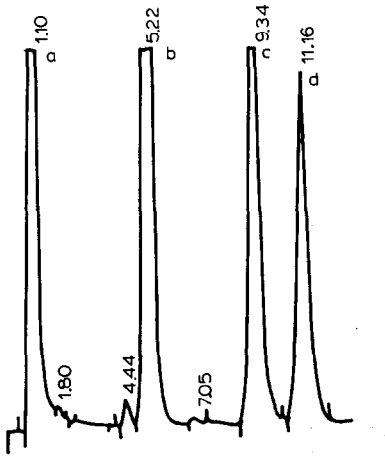

Fig. 2. Separation of anthracyclines by liquid chromatography. Column: 120 mm × 4.6 mm Nucleosil 50 5 μm. Mobile phase: methanol–water–buffer pH 2.5–dichloromethane (130:6.5:0.75:863). Flow-rate: 1.5 ml/min. Chart speed: 5 mm/min. Peaks: a = daunorubicin aglycone; b = daunorubicin; c = doxorubicin; d = duborimycin.

dodecyl sulphate as ion-pairing agent. The method was tried as described but the retention times were very long, as found by other workers[13]. By increasing the concentration of organic modifier the retention times of those substances were shortened (Fig. 3).

For daunorubicin it was considered that a 50:50 mixture of acetonitrile–water was optimal for the separation from the other anthracyclines (Fig. 4). The capacity factors and separation coefficient were calculated and are presented in Table V. The commercial batches of daunorubicin were then analysed and the percentage composition calculated by peak area "normalization" (Table VI). Using this system three

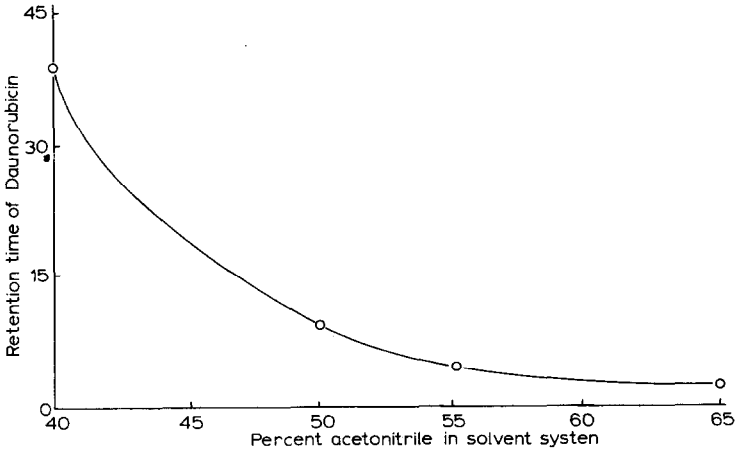

Fig. 3. Relationship between the percentage of organic modifier in the solvent system and the retention time of daunorubicin.

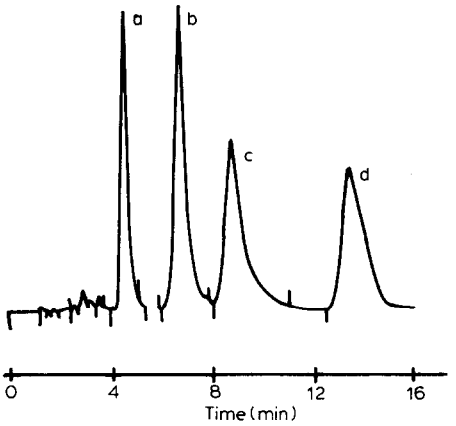

Fig. 4. Separation of anthracyclines by liquid chromatography. Column: 250 mm × 4.6 mm LiChrosorb RP-18. Mobile phase: acetonitrile–water (50:50) containing 0.01 *M* sodium dodecyl sulphate and 0.02 *M* phosphoric acid. Flow-rate: 2.0 ml/min. Chart speed: 5 mm/min. Peaks: a = daunorubicin aglycone; b = doxorubicin; c = duborimycin; d = daunorubicin.

TABLE V

CALCULATED CAPACITY FACTORS AND SEPARATION FACTORS FOR A MIXTURE OF ANTHRACYCLINES OBTAINED BY HPLC

Reversed-phase column: LiChrosorb RP-18, 250 mm × 4.6 mm. Mobile phase: acetonitrile–water (50:50) containing 0.01 *M* sodium dodecyl sulphate and 0.02 *M* phosphoric acid.

Substance	*k'*	α
Daunorubicin aglycone	2.54	1.7
Doxorubicin	4.31	1.4
Duborimycin	5.9	1.6
Daunorubicin	9.7	

TABLE VI

HPLC ANALYSIS OF BATCHES OF DAUNORUBICIN

Reversed-phase column: LiChromosorb RP-18, 250 mm × 5.6 mm. Mobile phase: acetonitrile–water (50:50) containing 0.01 *M* sodium dodecyl sulphate and 0.02 *M* phosphoric acid. Determination carried out by peak area "normalization" procedure. ND = Not detected; SMP = shoulder on main peak, not integrated.

Substance	*Batch number*	
	107	*114*
Daunorubicin aglycone	0.50	1.20
Unknown x	0.25	ND
Doxorubicin	0.42	0.32
Duborimycin	1.23	0.90
Unknown y	0.06	SMP
Daunorubicin	95.32	97.03
Unknown z	0.41	0.43

TABLE VII

DETERMINATION OF DAUNORUBICIN CONTENT (%) OF RAW MATERIAL BY DIFFERENT LABORATORIES USING DIFFERENT OCTYLDECYLSILYL COLUMNS (FIRST COLLABORATIVE TRIAL)

Laboratory	*Column*	*Raw material*	
		A	*B*
1	μBondapak C_{18}	96.50	91.6
2	Nucleosil C_{18}	96.3	–
3	Partisil ODS	–	91.6
4	Nucleosil C_{18}	96.4	–
5	Hypersil ODS	97.8	93.61
6	LiChrosorb RP-18	96.7	92.3
Mean		96.74	92.28
Coefficient of variation (%)		0.63	1.02

unknown peaks were observed compared with only two in the normal phase silica method.

In the light of those preliminary results with the reversed-phase, ion-pairing system it was decided to proceed to a small collaborative trial in which two of the participating laboratories were manufacturers of anthracyclines and as such had much experience to draw upon. Each laboratory examined two batches, in duplicate, of daunorubicin against a standard of known purity from Table VII it is seen that the results obtained are in good agreement. However, one laboratory expressed dissatisfaction with the column used (Nucleosil 5 C_{18}) since in their opinion there was an irregular resolution of the impurities from one batch of the stationary phase to another. Such an observation had already been made by other workers for the separation of other groups of compounds by steroids[14], cephalosporins[15] and polycyclic hydrocarbons[16,17]. As pointed out by Wouters *et al.*[15], a given separation on one

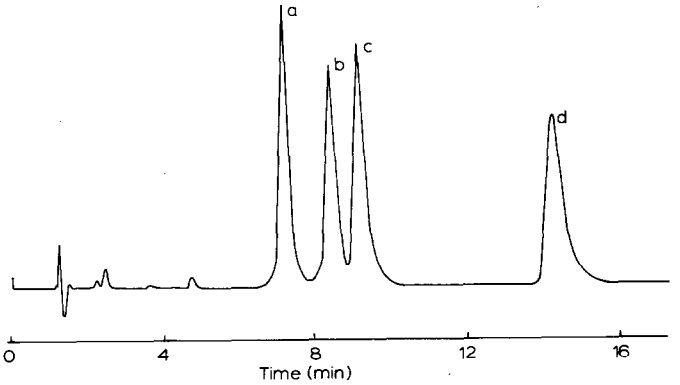

Fig. 5. Separation of anthracyclines by liquid chromatography. Column: 120 mm × 4 mm Hypersil ODS. Mobile phase: acetonitrile–methanol–water (45:5:50) containing 0.01 *M* sodium lauryl sulphate and 0.02 *M* phosphoric acid. Flow-rate: 2.0 ml min^{-1}. Chart speed: 20 mm/min. Peaks: a = epirubicin; b = doxorubicin; c = duborimycin; d = daunorubicin.

TABLE VIII

DETERMINATION OF DAUNORUBICIN CONTENT (%) OF RAW MATERIAL BY DIFFERENT LABORATORIES (SECOND COLLABORATIVE TRIAL)

Laboratory	*Column*	*Content*
1	Partisil 5-ODS-3	97.0
2	Partisil 5-ODS-3	97.2
3	Partisil 5-ODS-3	97.3
4	Hypersil ODS (5 μm)	97.7
Mean		97.3
Coefficient of variation (%)		0.30

column cannot necessarily be reproduced on another of the same type from another manufacturer or from a different batch of material from the same manufacturer. In such a case it is necessary to introduce a suitability test using at least two reference substances or a standard mixture to test for adequate separation before continuing with the analysis. This had been forseen in the experimental protocol; nonetheless the problem of varying resolution was still encountered but with apparent negligible effect on the calculated purity. As a result of this observation this laboratory suggested a slight change to the mobile phase (incorporation of methanol) which appeared to overcome the problem (Fig. 5). The linearity of regression was confirmed for the peak area against the concentration of daunorubicin and the regression coefficient for successive injections was 0.4% ($n = 8$). Subsequently another collaborative trial was conducted with one batch of daunorubicin against a standard of known purity, the results of which are presented in Table VIII. A coefficient of variation of 0.3% was obtained from the results submitted by the four laboratories. This slightly modified system is recommended as a suitable method for the assay of daunorubicin, and may appear in a monograph on daunorubicin hydrochloride in the European Pharmacopoeia.

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