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HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY AND THIN-LAYER CHROMATOGRAPHY OF ANTHRACYCLINE ANTIBIOTICS

SEPARATION AND IDENTIFICATION OF COMPONENTS OF THE DAUNORUBICIN COMPLEX FROM FERMENTATION BROTH*

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SUMMARY

A new solvent system composed of methanol–acidic water (pH 2.0 with phosphoric acid) has been developed for high-performance liquid chromatography of the daunorubicin complex from fermentation broth on a μBondapak C_{18} column. Application of this solvent system in conjunction with thin-layer chromatography in the identification of anthracycline antibiotics from the fermentation broth is discussed.

INTRODUCTION

High-performance liquid chromatography (HPLC) and thin-layer chromatography (TLC) have become two of the most important techniques for the separation, identification and isolation of synthetic and natural products. Both methods have certain limitations but, in conjunction, they complement each other.

This paper discusses HPLC and its application, in conjunction with TLC, for the identification of anthracyclines formed during the microbial production of daunorubicin (1) (NSC-82151).

Anthracycline antibiotics[1–4], in particular daunorubicin (also known as rubidomycin, daunomycin and rubomycin)[4–10], adriamycin (2) (NSC-123127; also known as doxorubicin)[11–13] and carminomycin I (3)[14,15] have aroused considerable interest in recent years because of their impact on cancer chemotherapy. Of particular significance is their potency against acute lymphocytic and myelogenous leukemias[16–18]; they are also active against a broad spectrum of solid tumors[13,19,20]. However, restricting their usefulness, is their dose-limiting cardiotoxicity[21,22].

In an attempt to eliminate, or at least to minimize, toxic side effects (especially cardiotoxicity) and to increase the clinical usefulness of these antibiotics, various structural modifications have been carried out[4]. Because of the demand for and the

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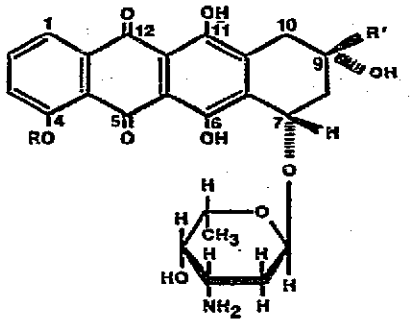


1: Daunorubicin: R=CH_3, R'= $-\overset{O}{\overset{\|}{C}}-CH_3$

2: Adriamycin: R=CH_3, R'= $-\overset{O}{\overset{\|}{C}}-CH_2OH$

3: Carminomycin I: R=H, R'= $-\overset{O}{\overset{\|}{C}}-CH_3$

cost of producing these products, efforts have also been directed towards strain development for higher yields. During this program at the Frederick Cancer Research Center[23,24], it was necessary to identify daunorubicin precursors and co-metabolites. Based on this knowledge, fermentation conditions could then be modified to enhance daunorubicin production.

TLC has been used to monitor daunorubicin and other anthracyclines in fermentation broths, but there are certain limiting factors, and often more than one mobile phase must be used for the TLC[25–27]. In order to eliminate the use of multiple mobile phases in TLC, and also because of the high sensitivity of HPLC, it was decided to develop an HPLC system that could be used to monitor daunorubicin fermentation, determine the number of components in a particular fermentation, and, finally, quantitate daunorubicin and/or its precursors and co-metabolites.

EXPERIMENTAL

General

Daunorubicin, daunorubicinone, 7-deoxydihydrodaunorubicinone, ε-rhodomycinone, glycoside I (baumycin A_2), 30-8-1M (an unknown) and other similar compounds used in the present investigation were purified by column chromatography and preparative TLC on silica gel. Their identities were confirmed by ultraviolet–visible (UV–VIS), infrared (IR), proton nuclear magnetic resonance (^{1}H NMR), carbon magnetic resonance (^{13}C NMR), electron-impact mass spectrometry (EI-MS) and field-desorption mass spectrometry (FD-MS).

All chemicals were of analytical grade and were used without further purification.

The mobile phases were prepared from methanol, acetonitrile [both from Burdick and Jackson (Muskegon, MI, U.S.A.), distilled in glass], PIC B-7 (Waters

Assoc., Milford, MA, U.S.A.), phosphoric acid (Mallinckrodt, St. Louis, MO, U.S.A.; analytical grade) and glass-distilled water.

All HPLC work was carried out on a μBondapak C_{18} column using a UV detector (254 nm; 0.04 a.u.f.s.).

Apparatus

A Waters Assoc. Model 6000A solvent-delivery system, with Model 660 solvent programmer, a Waters Assoc. U6K universal injector with a sample loop of 2 ml and a Schoeffel SF 770 Spectroflow variable-wavelength detector were used for all HPLC work. The detector was set at 254 nm and 0.04 a.u.f.s. (cell volume 8 μl; path length 10 mm). The μBondapak C_{18} columns (30 cm × 3.9 mm I.D.; particle size 10 μm) used were manufactured by Waters Assoc.

A London Co. pH meter (The London Co., Cleveland, OH, U.S.A.), Model 64, equipped with a Sensorex (Sensorex, Westminster, CA, U.S.A.) electrode was used for pH measurements. TLC plates used were of pre-coated silica gel 60 F-254 (Merck, Darmstadt, G.F.R.). Millipore filters (type HA, pore size 0.45 μm and FH, pore size 0.5 μm) (Millipore, Bedford, MA, U.S.A.) were used for filtering solvents and samples.

Preparation of mobile phase

The glass-distilled water was adjusted to pH 2.0 with phosphoric acid and was filtered through a Millipore filter (type HA); methanol or acetonitrile was also filtered through a Millipore filter (type FH). Measured amounts of the organic and aqueous phases were mixed by stirring. The PIC B-7 solvent system was also prepared in a similar manner. Filtration was avoided, after mixing the solvents, to eliminate the possibility of a change in ratio of low- and high-boiling solvents (see Results and discussion).

Preparation of samples for HPLC injection

Authentic samples of daunorubicin, daunorubicinone, ε-rhodomycinone, baumycin A_2 and other structurally related compounds were carefully weighed and dissolved in methanol (*ca.* 15 mg/l). The solutions were filtered through Millipore filters (type FH), and 10-μl aliquots were injected onto the HPLC column.

Preparation of samples for TLC

Standard samples of daunorubicin, daunorubicinone, ε-rhodomycinone, baumycin A_2, 7-deoxydihydrodaunorubicinone and others were taken in *ca.* 1–2 ml of methanol or chloroform to give pale orange-colored solutions. These solutions were applied in a range of 2 μl to 20 μl to give well-defined spots on development. Butanol extracts from whole-broth samples were spotted without further preparation; generally, 20–30 μl of butanol extract were necessary in order to see all the compounds present.

Extraction of fermentation broth

General procedure. In a typical experiment, the whole broth is mixed with an equal volume of 1-butanol; the pH of the mixture is adjusted to 8.5 with 50% aqueous sodium hydroxide solution, then the mixture is stirred vigorously for 30 min and

filtered through Celite. Aqueous and butanol layers are separated, and the aqueous layer and cake are mixed and re-extracted with fresh 1-butanol. The combined butanol extracts are washed with water, then the pH is adjusted to 5.5 with aqueous 6 *N* hydrochloric acid, and the butanol is concentrated under vacuum at *ca.* 40°C. The concentrate is then precipitated with heptane, filtered, and dried under high vacuum to yield the crude daunorubicin complex, which is analysed by TLC and HPLC. This procedure is summarized in Fig. 1.

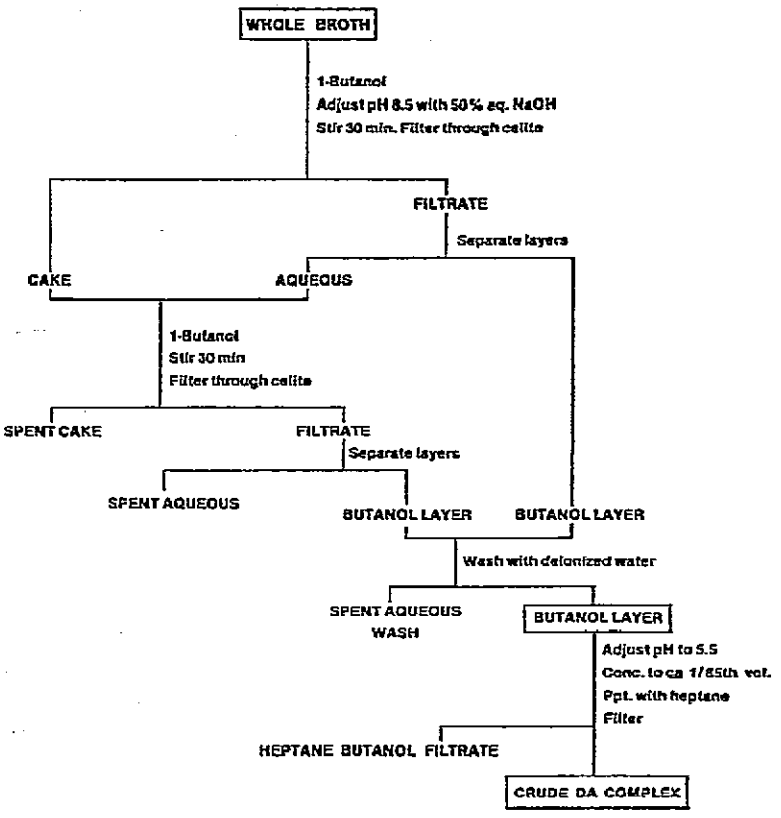


Fig. 1. General procedure for isolation of total crude daunorubicin complex from fermentation broth.

The above general procedure has been modified, and, for most of the work, the following three procedures (summarized in Fig. 2) were developed for the partial or total extraction of the fermentation broth for HPLC work.

(A). Whole broth is adjusted to pH 8.5 and stirred with 1-butanol for 15 min. Spent cake is removed by filtration, and the butanol layer is separated and filtered through a Millipore filter (type FH); the filtrate is injected onto the HPLC column to determine the composition of the unhydrolysed broth (Fig. 2A).

(B). Whole broth is mixed with 1-butanol and adjusted to pH 1.5 with hydrochloric, sulphuric or oxalic acid, with stirring. The mixture is heated at 40°C for 30 min, then cooled to room temperature, and its pH is adjusted to 8.5 with aqueous

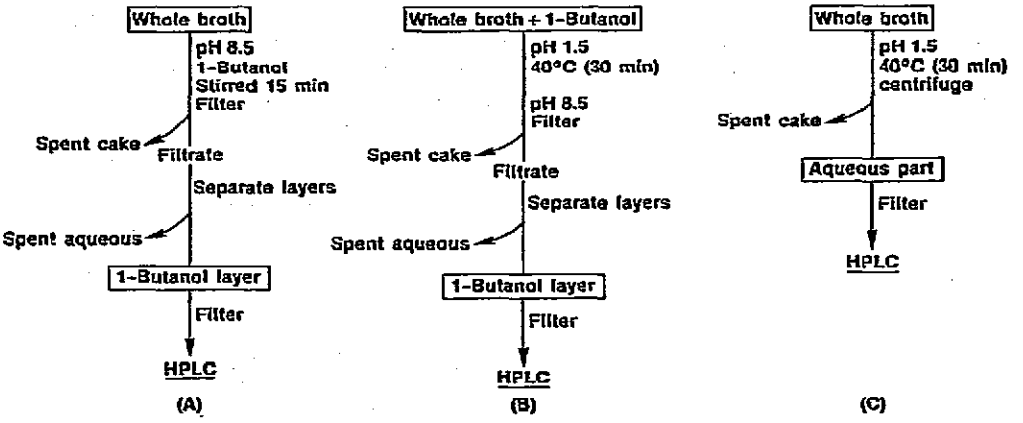


Fig. 2. Modified procedures for extraction of daunorubicin fermentation broth under different conditions.

sodium hydroxide. The spent cake is removed by filtration, and the butanol layer is separated, filtered through a Millipore filter (type FH), and injected onto the HPLC column to determine the composition of the hydrolysed broth (Fig. 2B).

(C). Whole broth is adjusted to pH 1.5 with aqueous hydrochloric, sulphuric or oxalic acid and heated at 40°C for 30 min. The broth is then centrifuged, and the aqueous part is filtered through a Millipore filter (type HA) and injected onto the HPLC column to determine the daunorubicin content (Fig. 2C).

RESULTS AND DISCUSSION

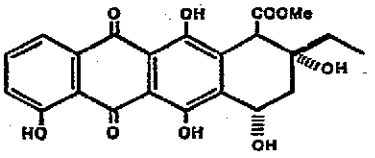
In the course of these studies, it was necessary to identify and determine the relative percentages of the different components in a particular fermentation.

TLC has been used for the separation of anthracycline antibiotics, but quantitation has been difficult because of the complexity of the fermentation mixture[25–27]. HPLC systems have been reported in the literature[28–33] for such clinically useful anthracyclines as daunorubicin and adriamycin, and their metabolites, but they have not proved useful for complex mixtures.

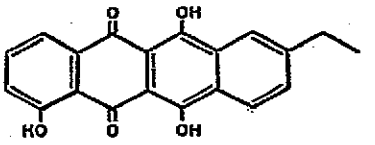
The present investigation was directed toward development of one or more simple HPLC and TLC systems for the separation, quantitation and identification of various components in a daunorubicin fermentation broth. The daunorubicin is produced as its higher glycosides, along with other anthracyclines, during the course of a fermentation. Subsequently, the higher glycosides are partially hydrolysed, and the resulting daunorubicin is then purified by solvent extraction at different pH values and crystallized[24].

In order to determine the peak production of the glycosides that give rise to daunorubicin, samples are taken at different time intervals, worked up and hydrolysed (or *vice-versa*), and assayed for daunorubicin in the broth by HPLC[33] or TLC[27].

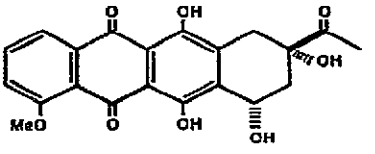
Presently known methods (HPLC and TLC) are suitable for such quantitation if the assay samples contain two or three components. In any crude mixture, it is difficult to quantitate exactly when known HPLC mobile phases are used, because of



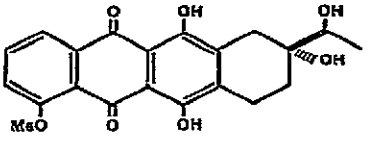
4: ε-Rhodomycinone



5: Descarbomethoxybisanhydro-ε-rhodomycinone



6: Daunorubicinone



7: 7-Deoxydihydrodaunorubicinone

the overlap of certain peaks. Further, in order to determine the composition of a fermentation broth at various time intervals or after certain genetic manipulations, it became necessary to separate the components. TLC in various solvents, particularly in chloroform–methanol–formic acid (80:20:2) and hexanes–chloroform–methanol (5:5:1), always indicated (Fig. 3) that the whole broth was a complex mixture of at least eleven to fifteen components. Some of these, ε-rhodomycinone (4), descarbomethoxybisanhydro-ε-rhodomycinone (5), daunorubicinone (6) and 7-deoxydihydrodaunorubicinone (7) were isolated (Table I and Fig. 4) by repeated silica gel column chromatography and preparative TLC. Because of the close proximity of several spots

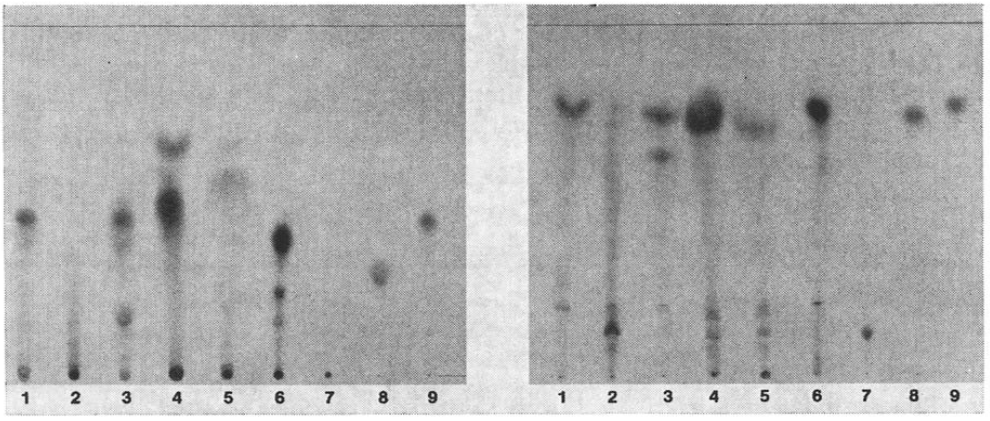


Fig. 3. Thin-layer chromatograms of various butanol extracts. Plate: silica gel G (20 × 20 cm, 0.25 mm thick). Solvent systems: left, chloroform–methanol–formic acid (80:20:2); right, chloroform–hexanes–methanol (5:5:1). 1 = Total butanol extract of whole daunorubicin broth; 2 = butanol extract after acid hydrolysis; 3 = total butanol extract of 2-week-old broth; 4 = total butanol extract of 30-8 broth; 5 = total butanol extract of DAMCT-1 broth; 6 = total ethanol extract of A-21 cake; 7 = daunorubicin; 8 = daunorubicinone; 9 = ε-rhodomycinone.

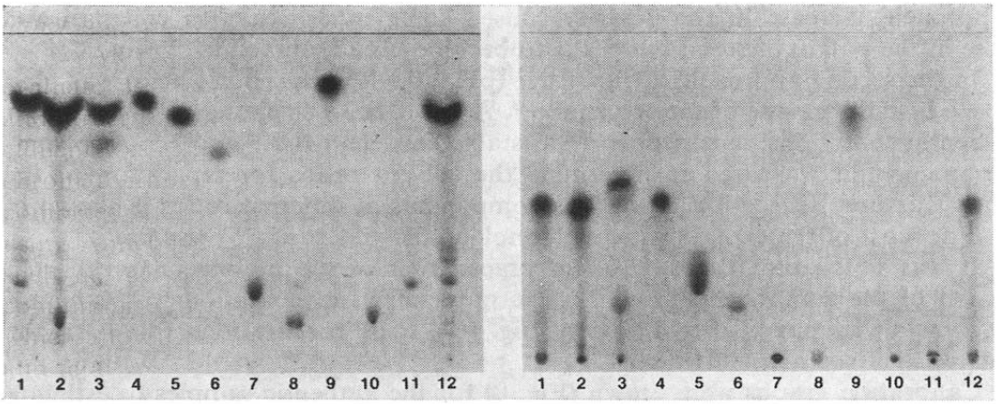


Fig. 4. Thin-layer chromatograms of various isolated anthracyclines. Plates as in Fig. 3. Solvent systems: left, chloroform–methanol–formic acid (80:20:2); right, chloroform–heptane–methanol (5:5:1). 1 and 12 = DAF-33; 2 = DAF-33-1; 3 = DAF-33-2, 4 = ε-rhodomycinone; 5 = daunorubicinone; 6 = 7-deoxydihydrodaunorubicinone; 7 = glycoside I (baumycin A_2); 8 = 30-8-1M (unknown); 9 = descarbomethoxybisanhydro-ε-rhodomycinone; 10 = daunorubicin; 11 = DAMCT-1-1 (unknown).

in TLC (Fig. 4), quantitation was difficult, although TLC provided an excellent qualitative analytical method. In order to develop an efficient mobile phase for separation of the daunorubicin complex by HPLC, preliminary investigations were started on a synthetic mixture (prepared from pure components isolated from daunorubicin fermentation broth) with the known solvents used for assay of daunorubicin. The separation of such a mixture on a μBondapak C_{18} column with methanol–water–PIC B-7 (65:35:1.8) is shown in Fig. 5. This mobile phase did not separate daunorubicin and 7-deoxydihydrodaunorubicinone, both of which are daunorubicin fermentation co-metabolites, under different conditions[34]. Thus, we decided to change or modify the solvent system.

Three approaches were used: (1) changing the solvent ratio, (2) replacing the PIC B-7 reagent, and (3) replacing both the PIC B-7 and the organic solvent. In the

TABLE I

THIN-LAYER CHROMATOGRAPHIC BEHAVIOUR OF SOME ANTHRACYCLINES ISOLATED FROM DAUNORUBICIN FERMENTATION BROTH

Compound No.	*Anthracycline*	*Structure No.*	R_F *value**	
			Solvent A	*Solvent B*
1	ε-Rhodomycinone	4	0.79	0.48
2	Daunorubicinone	6	0.74	0.25
3	7-Deoxydihydrodaunorubicinone	7	0.63	0.16
4	Glycoside I (baumycin A_2)	8	0.21	0.00
5	30-8-1M	Unknown	0.12	0.00
6	Descarbomethoxybisanhydro-ε-rhodomycinone	5	0.83	0.74
7	Daunorubicin	1	0.15	0.00
8	DAMCT-1-1	Unknown	0.23	0.03

* Silica gel plates (20 × 20 cm; 0.25-mm layer). Solvent A, chloroform–methanol–formic acid (80:20:2); solvent B, chloroform–heptane–methanol (5:5:1).

first approach, increase in the polarity caused some other peaks to collapse, and decrease in the polarity caused the peaks to become broad, and elute slowly.

In the second approach, acidic water (pH 2.0 with phosphoric acid) was used to replace PIC B-7 as one of the solvents[32,33]. A pH of 2.0 was chosen because, under experimental conditions, daunorubicin was stable between pH 1.5 and 2.5. A solvent-programming unit was used to determine the solvent ratios for separation of the authentic mixture. Separation of various components at different ratios is shown in Fig. 6. For most of the experiments, methanol–acidic water (65:35) was used.

It was noted that the method of preparation of the mobile phase affected separation of the components (Fig. 7). The most effective separation was achieved when solvents were mixed after filtration (Fig. 7C). In all preparations, therefore, the solvents were mixed after filtration.

Calibration graphs were drawn (Fig. 8) for the authentic samples to estimate the amount of the components in the fermentation broth.

When the organic solvent was replaced by acetonitrile and used with acidic water (pH 2.0 with phosphoric acid) in different ratios, poorer separations were observed (compare Figs. 7 and 9). The best separation in this solvent was obtained at a acetonitrile–acidic water ratio of 30:70, as shown in Fig. 9.

As a result of these experiments, it was concluded that the mobile phase containing methanol–acidic water (pH 2.0 with phosphoric acid) (65:35) was the best

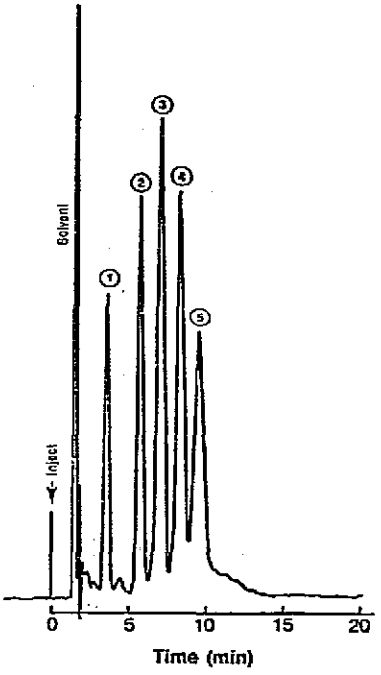


Fig. 5. High-performance liquid chromatogram of a mixture of six anthracyclines. Mobile phase: methanol–water–PIC B-7 (65:35:1.8); flow-rate, 2 ml/min. Peaks: 1 = 30-8-1M; 2 = daunorubicinone; 3 = daunorubicin and 7-deoxydihydrodaunorubicinone; 4 = ε-rhodomycinone; 5 = baumycin A_2.

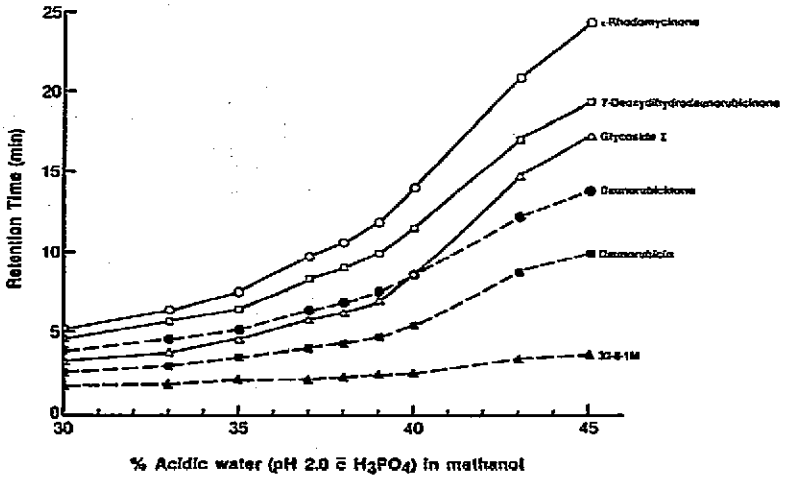


Fig. 6. Plot of HPLC retention times, on a μBondapak C_{18} column (30 cm × 3.9 mm I.D.), of various anthracyclines isolated from daunorubicin fermentation broth *vs.* acidic water (pH 2.0 with phosphoric acid)–methanol at different ratios.

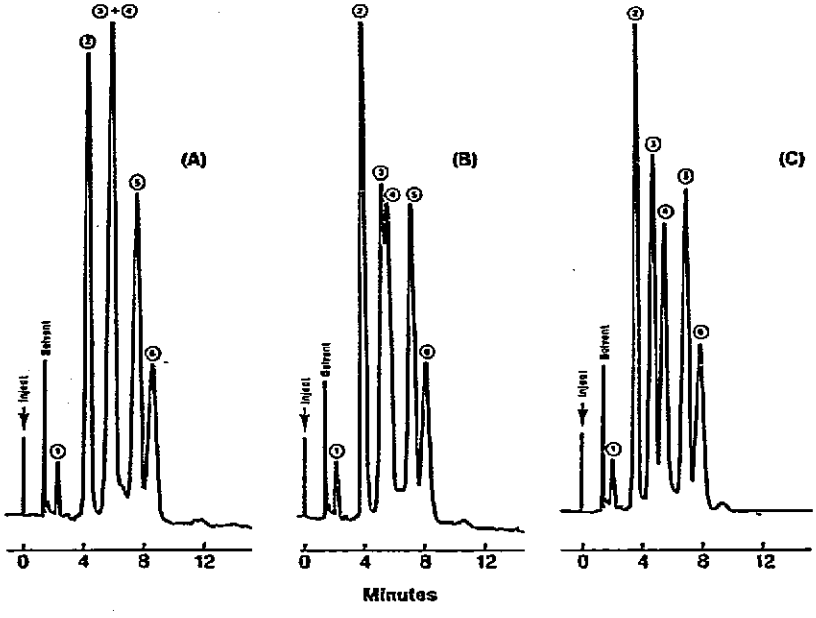


Fig. 7. Effect of solvent preparation on separation of various anthracyclines; mobile phase, methanol–water (pH 2.0 with phosphoric acid); flow-rate, 2 ml/min. Method of solvent preparation: A, mixed and filtered immediately; B, mixed and filtered after some time; C, filtered and then mixed. Peaks: 1 = 30-8-1M (unknown); 2 = daunorubicin; 3 = baumycin A_2; 4 = daunorubicinone; 5 = 7-deoxydihydrodaunorubicinone; 6 = ε-rhodomycinone.

for separation of daunorubicin and its co-metabolites. It was also simple and inexpensive compared with other solvent systems reported in the literature[28–33].

A comparison of HPLC data for butanol extracts of daunorubicin fermentation

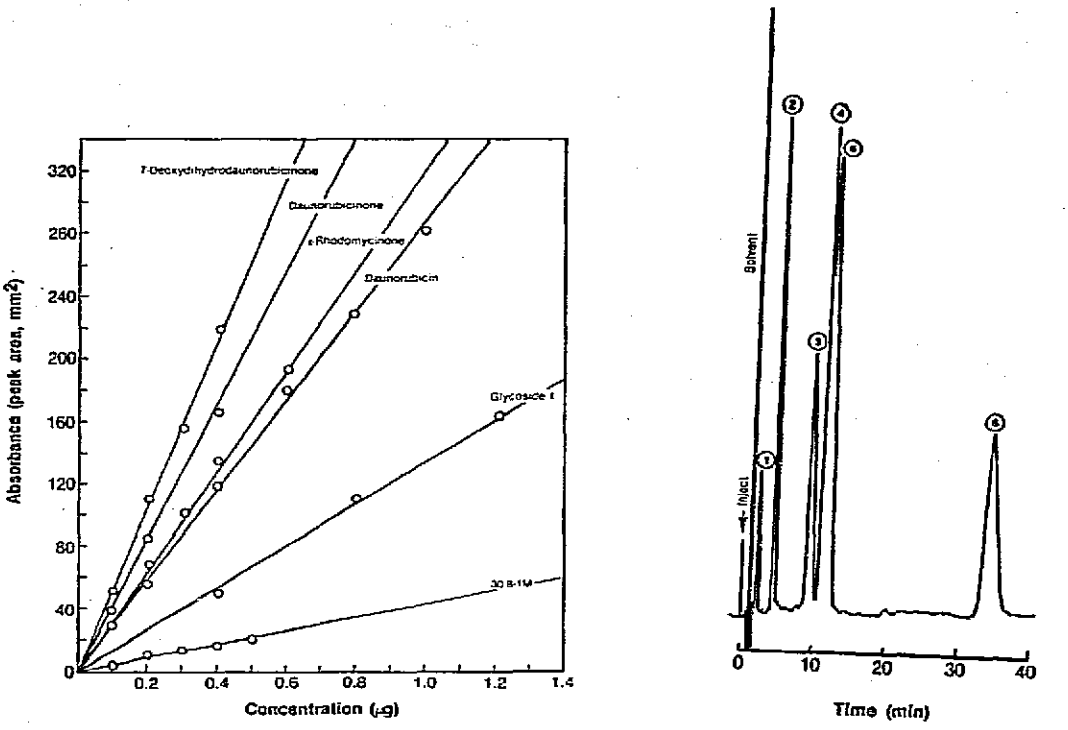


Fig. 8. Calibration graphs for authentic anthracyclines. Mobile phase: methanol–acidic water (pH 2.0 with phosphoric acid) (65:35).

Fig. 9. High-performance liquid chromatogram of six anthracyclines; mobile phase: acetonitrile–acidic water (pH 2.0 with phosphoric acid) (30:70); flow-rate: 3 ml/min. Peaks: 1 = 30-8-1M; 2 = daunorubicin; 3 = baumycin A_2; 4 = daunorubicinone; 5 = 7-deoxydihydrodaunorubicinone; 6 = ε-rhodomycinone.

broth at various time intervals is shown in Table II. From these data, it is evident that little daunorubicin is formed in fermentation; most occurs as its higher glycoside I, identified[35] as baumycin A_2 (8; ref. 36). Further, there is not much difference in the products formed after 140 h. Table II also shows that the ratio of ε-rhodomycinone

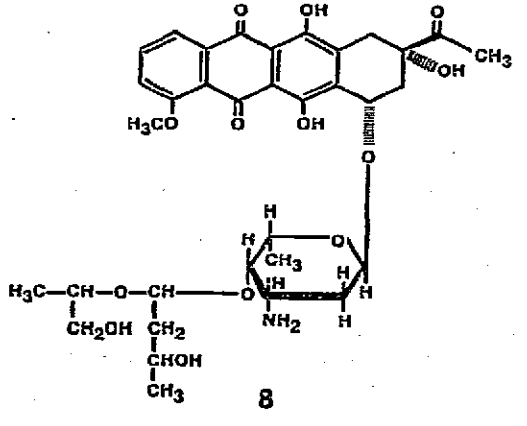


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TABLE II

MONITORING OF PERCENTAGE COMPOSITION OF DAUNORUBICIN FERMENTATION AT VARIOUS TIME INTERVALS

Peak Nos.	*Retention time** (min)*	*Assignment*	*Composition (%) of DAF-37 broth**						
			46 h	*70 h*	*94 h*	*140 h*	*178 h*	*214 h*	*238 h*
1	2.6		4.1	2.9	2.9	1.1	1.6	1.0	1.2
2	3.3	Daunorubicin	8.6	12.1	3.9	2.8	3.7	3.8	3.6
3	4.0								
4	4.8	Glycoside I			10.8	15.5	16.0	15.8	15.2
5	5.8	Daunorubicinone	4.5	8.3	10.2	6.5	5.7	5.7	5.3
6	6.2								
7	7.1	7-Deoxydihydro-daunorubicinone	2.1	1.4		2.9	1.9	1.8	1.6
8	8.0	ε-Rhodomycinone	11.9	25.7	32.8	33.7	30.3	28.5	33.8
9	9.6		6.9	5.8	3.8	3.7	2.9	3.5	2.5
10+11+12 +13	10.4, 11.1, 12.7, 13.8		7.0	5.4	2.6	7.4	6.2	7.2	6.3
14	15.2		8.5	5.3	4.8	3.7	4.2	4.1	3.7
15	16.0		9.8	7.8	5.7	3.9	5.0	5.0	4.7
18	24.8		9.1	6.1	4.8	3.8	5.2	4.7	3.9
19	26.6		11.4	11.2	5.1	4.3	6.1	6.1	5.6
Other peaks	26.6		15.9	6.7	12.4	10.7	11.1	12.7	12.4

* Calculated from the HPLC profile of total butanol extract.

** On a μBondapak C_{18} column (30 cm × 3.9 mm I.D.); mobile phase: methanol–acidic water (pH 2.0 with phosphoric acid) (65:35); flow-rate: 2 ml/min.

to baumycin A_2 at 94 h changes after 140 h; it then remains constant. The change in this ratio could have occurred through some of the ε-rhodomycinone being converted into baumycin A_2 or, after ε-rhodomycinone plateaued, other components were transformed into baumycin A_2. Isolation and characterization of the various components (which are in progress in this laboratory) would confirm either or both possibilities. A comparison of actual HPLC traces at 46, 94 and 238 h and the assignments of various peaks are shown in Fig. 10. From these data, it is very clear that peak 5 (assigned as daunorubicinone) and peak 6 (an unknown) go to peak 4 (assigned as baumycin A_2).

Fig. 11 shows a comparison of a butanol extract of a daunorubicin fermentation broth (batch DAF-33) harvested after 166 h and worked up according to Fig. 2A (top chromatogram) with the acid-treated (pH 1.5) broth (middle chromatogram) and the broth stored at pH 7.5 for 2 weeks (lower chromatogram). These chromatograms clearly indicate that baumycin A_2 (peak 4), as expected, goes to daunorubicin (peak 2) on acid treatment, and, on storage at neutral pH, baumycin A_2 is almost completely converted into 7-deoxydihydrodaunorubicinone.

Fig. 12 shows application of this mobile phase in daunorubicin assay. Two sample broths, Nos. 3508 and 4491, were extracted as described in Fig. 2C and were injected onto the HPLC column using methanol–acidic water ratios of 65:35 (upper chromatograms) and 60:40 (lower chromatograms).

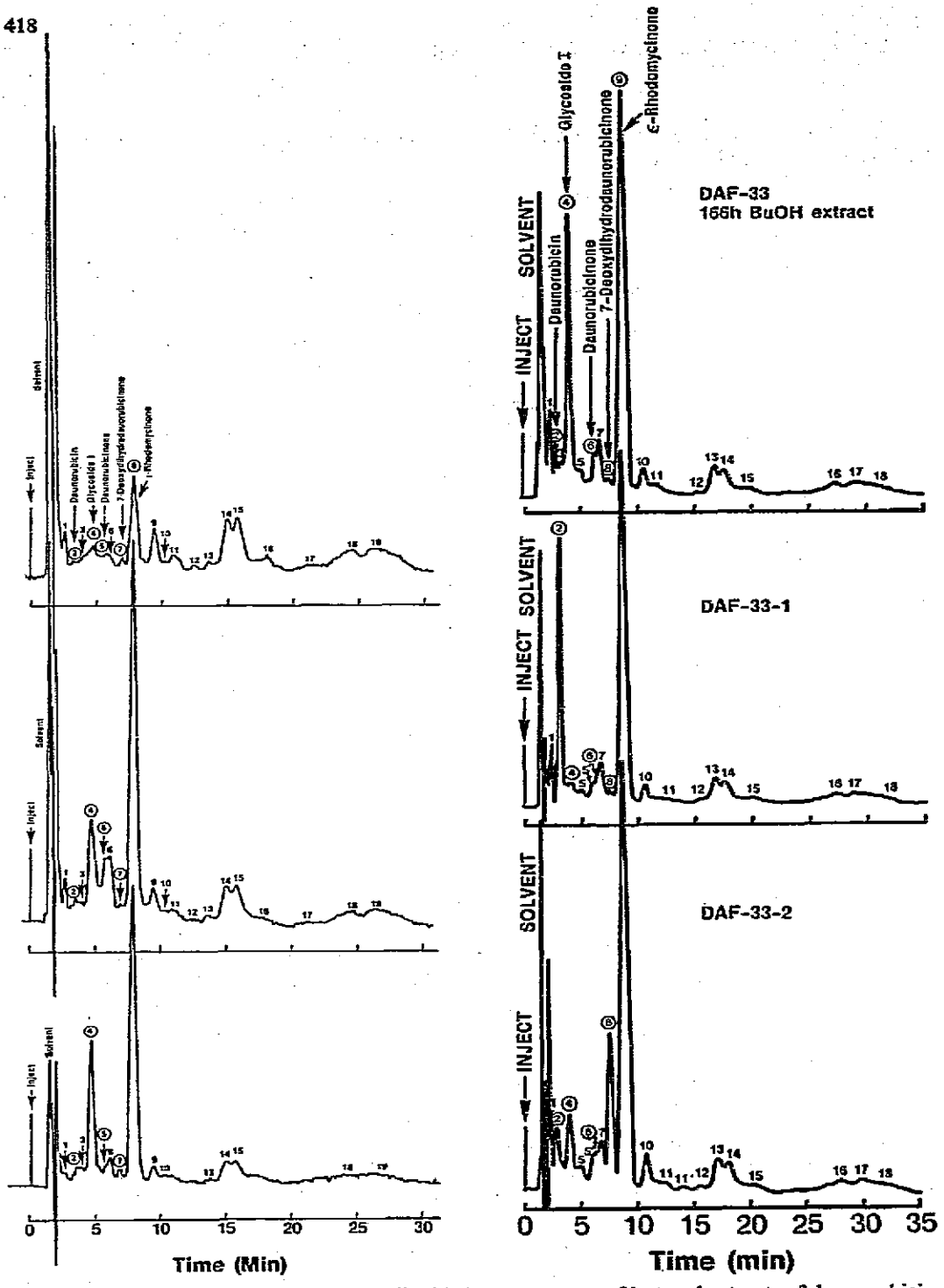

Fig. 10. Comparison of high-performance liquid chromatograms of butanol extracts of daunorubicin fermentation broth (batch DAF-37) at three time intervals: 46 h (top), 94 h (middle) and 238 h (bottom). Mobile phase as in Fig. 8; flow-rate, 2 ml/min.

Fig. 11. High-performance liquid chromatograms of butanol extracts of daunorubicin fermentation broth (batch DAF-33). Top: extracted at pH 8.5 (Fig. 2A); middle: extracted after hydrolysis (Fig. 2B); bottom: extracted as in Fig. 2A after 2 weeks; mobile phase and flow-rate as in Fig. 10.

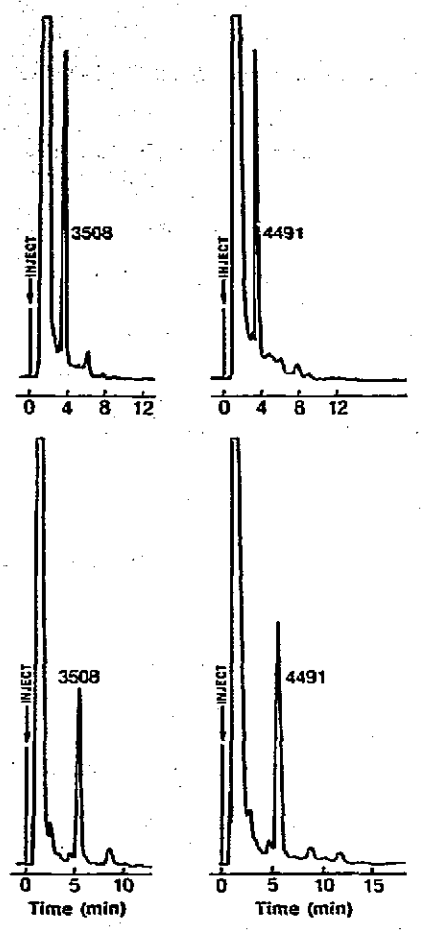

Fig. 12. High-performance liquid chromatograms of extracts of daunorubicin fermentation broths (samples 3508 and 4491) obtained according to Fig. 2C. Mobile phase: top, methanol–pH 2.0 water (65:35); bottom, methanol–pH 2.0 water (60:40); flow-rate, 2 ml/min.

CONCLUSION

We have thus developed a simple, fast and inexpensive HPLC solvent system for the separation of anthracycline antibiotics in daunorubicin fermentation broths; its application to monitoring fermentation broth, identifying various peaks and assaying daunorubicin is discussed.

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