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Solid-phase extraction and optimized separation of doxorubicin, epirubicin and their metabolites using reversed-phase high-performance liquid chromatography*

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Abstract: A reversed-phase isocratic high-performance liquid chromatographic method is described in which a formal structured procedure was applied to predict the mobile phase composition giving optimal baseline resolution of the clinically important anticancer agents doxorubicin and 4′-epidoxorubicin (epirubicin), their principal metabolites, and daunorubicin (internal standard). These formal statistical procedures included the simultaneous techniques of solvent selectivity triangle and factorial design for range-finding preliminary studies, followed by use of the modified simplex, a sequential procedure. These were used to select the parameters of organic modifier, buffer strength and pH necessary for use with a Spherisorb ODS 1 column, to achieve optimal separation of eight anthracycline solutes. Ultraviolet and fluorescence detection was used (λ_{ex} = 254 nm, λ_{em} = 560 nm), and the latter gave a low detection limit for doxorubicin in serum of 1 ng ml^{-1}. The optimal mobile phase composition was determined to be acetonitrile–0.06 M Na_2 HPO_4 containing 0.05% (v/v) triethylamine adjusted to pH 4.6 with 0.03 M citric acid (35:65, v/v).

A solid-phase extraction method was developed to enable the selective isolation of anthracyclines by adsorption onto C_8 Bond-Elut cartridges, and is based on extraction of serum spiked with a mixture of the anthracycline solutes. The anthracyclines were eluted using acetonitrile–0.2 M Na_2 HPO_4 containing 0.05% (v/v) triethylamine adjusted to pH 3.6 with 0.1 M citric acid (67.5:32.5, v/v). Reproducible recoveries for doxorubicin (94 ± 8%) and for epirubicin (96 ± 8%) were obtained (n = 5). In particular, recoveries for the 7-deoxyaglycone metabolite (99%) were higher than other extraction methods cited. The solid-phase extraction method described enables rapid and reproducible determinations of these anthracyclines and their metabolites in biological matrices regardless of their disparate physicochemical properties.

Keywords: *Doxorubicin; epirubicin; anthracyclines; doxorubicinol; solid-phase extraction; formal statistical procedures.*

Introduction

The anthracycline group of antitumour antibiotics constitute a class of compounds with potent antineoplastic properties, and include doxorubicin (adriamycin), a clinically important anticancer agent which is effective against a broad spectrum of malignancies [1] and the antileukaemic agent, daunorubicin. The clinical value of these drugs is, however, limited by a potentially fatal cardiomyopathy which becomes clinically significant above a certain cumulative dose [2]. In addition there is evidence that doxorubicinol, the principal metabolite of doxorubicin, may also be cardiotoxic [3]. When anthracycline cardiotoxicity becomes clinically important, treatment with these agents must cease irrespective of the status of the disease [4], and as a result many research groups have synthesized related analogues of doxorubicin in attempts to find active candidates which circumvent this toxicity. These analogues include 4′-epidoxorubicin (epirubicin) which is reported to be as potent an antitumour agent as doxorubicin, though generally less cardiotoxic [5]. Doxorubicin and epirubicin are extensively metabolized in the liver, and the main metabolic pathway involves C-13 carbonyl reduction by aldo–keto reductases to yield doxorubicinol and epirubicinol with further metabolism resulting in the formation of the 7-hydroxy- and 7-deoxyaglycones (Fig. 1).

The current literature methods for the assay of anthracyclines in clinical samples regularly employ reversed-phase high-performance liquid chromatography (HPLC) as the analytical method of choice [6]. Normal-phase HPLC methods are unable to separate the relatively non-polar anthracycline aglycones, and ion-

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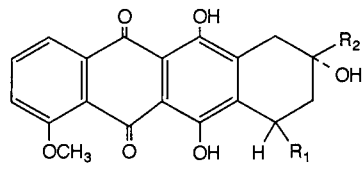
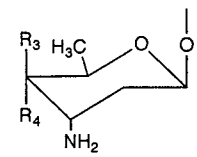


Anthracyclinone aglycone



Daunosamine sugar (D)

COMPOUND	R1	R2	R3	R4
Doxorubicin	D	$COCH_2OH$	H	OH
Epirubicin	D	$COCH_2OH$	OH	H
Doxorubicinol	D	$CHOHCH_2OH$	H	OH
Epirubicinol	D	$CHOHCH_2OH$	OH	H
Doxorubicinol aglycone	OH	$CHOHCH_2OH$	-	-
7-OH doxorubicin aglycone	OH	$COCH_2OH$	-	-
7-deoxydoxorubicin aglycone	H	$COCH_2OH$	-	-
Daunorubicin	D	$COCH_3$	H	OH

Figure 1
Structures of doxorubicin, epirubicin, their principal metabolites, and daunorubicin.

pair chromatography and gradient elution offer no significant advantage over simpler isocratic reversed-phase methods (reviewed in ref. 7). However, in some reversed-phase methods poor resolutions of parent anthracyclines and metabolites are described. Previously described anthracycline extractions have been achieved from biological tissues or fluids using an initial purification stage, e.g. solvent extraction or protein precipitation, followed by injection onto a reversed-phase HPLC system [8, 9].

In this study formal optimization procedures were applied to changes in the mobile phase composition in order to resolve eight anthracycline-related solutes including the antineoplastic agents doxorubicin and epirubicin, and their clinically important metabolites doxorubicinol, epirubicinol, doxorubicinol aglycone, 7-hydroxydoxorubicin aglycone and 7-deoxydoxorubicin aglycone using daunorubicin as internal standard (Fig. 1). Although both doxorubicin and epirubicin are unlikely to be used concomitantly in clinical use, these analogues share common metabolites and hence a universal HPLC method was sought to resolve parent drugs and their metabolites. Also described is a rapid and selective method for the separation of these solutes, with widely differing lipid solubilities, from biological matrices using a solid-phase extraction method followed by HPLC analysis.

Experimental

Materials and methods

Materials. All anthracyclines and related compounds were kindly donated by Farmitalia Carlo-Erba (Milan, Italy). Acetonitrile, methanol and tetrahydrofuran (THF) were HPLC grade, and all other chemicals were of analytical grade. Water was double distilled in glass. All analytical glassware in contact with drug solutions was silanized with Repelcote (BDH) and rinsed with methanol prior to use.

Sample preparation. Stock solutions of doxorubicin and epirubicin in water, and metabolites in methanol (all 10^{-4} M) were prepared, protected from light, stored at −20°C, and thawed immediately prior to use.

Liquid chromatography. The HPLC system consisted of a LKB 2150 pump, with either a Hewlett Packard 1046A fluorescence detector (Hewlett Packard, Waldbron, Germany) or a LDC/Milton Roy Spectromonitor 3000 variable wavelength UV-detector (LDC/Milton Roy, FL, USA), and a Hewlett Packard HP3396A integrator. UV detection at 254 nm was used for formal optimization procedures; fluorescence detection was used for analysis of drugs in biological matrices, using excitation and emission wavelengths of 254 and 560 nm, respectively. The stationary phase was 5 μm

Spherisorb ODS I with column dimensions either 150 mm (for optimization procedures) or 250 mm (for solid-phase extractions) × 4.6 mm i.d. (Phase Separations, Deeside, UK); an Alltech guard column with 10 × 4.6 mm i.d. cartridges was also used, containing the same stationary phase. The flow rate of mobile phase was 1 ml min^{-1}, and the injection volume was 20 μl. Mobile phases were initially based on a composition of acetonitrile–0.02 M NaH_2PO_4 buffer containing 0.1% (v/v) triethylamine adjusted to pH 4 with H_3PO_4 (35:65, v/v; mobile phase I). In mobile phases II and III, acetonitrile was replaced by methanol (43:57, v/v) and THF (25:75, v/v), respectively. Binary mixtures (1:1, v/v) of mobile phases I and II, I and III, and II and III produced mobile phases IV, V and VI, respectively. Mobile phase VII contained mobile phases I, II and III (1:1:1, v/v/v). Additional mobile phases were modified in factorial designs/simplex procedures, in which a new buffer system containing 0.05% (v/v) triethylamine was used, containing Na_2HPO_4 (in the range 0.05–0.2 M) adjusted to an appropriate pH with citric acid (in the range 0.025–0.1 M); the THF composition ranged from 0 to 5% (v/v), and the pH was adjusted between 3.2 and 5.2.

Recoveries. Recoveries were determined by the analysis of five independently prepared plasma samples containing all eight anthracycline/anthracyclinone compounds at concentrations in the range 50–500 ng ml^{-1}. The recoveries were calculated by comparison with standard solutions of the same drug concentration in elution solvent.

Formal optimization procedures

Solvent selectivity triangle. The seven mobile phases used for construction of the solvent selectivity triangle were prepared in 0.02 M NaH_2PO_4 (adjusted to pH 4.0 with H_3PO_4) and contained organic modifier with equivalent solvent strength to 35% (v/v) acetonitrile, calculated using the equations of Snyder [10]:

$$ST = s_a\psi_a + s_b\psi_b + \ldots,$$

where ST describes the total solvent strength of the mobile phase, s is the solvent strength weighting factor, and ψ is the volume fraction of the solvent. Using the s values of 2.6 for methanol, 3.2 for acetonitrile, 4.5 for THF and 0 for water, the proportions of organic modifier equivalent to 35% (v/v) acetonitrile were 43% (v/v) methanol, and 25% (v/v) THF.

The stationary phase (5 μm Spherisorb ODS 1 150 × 4.6 mm i.d.) was selected following previous studies which indicated the suitability of this column for anthracycline separations [11]. Mobile phases I–VII were each eluted through the column at 1.0 ml min^{-1} and the eluate monitored using UV detection as described above. Aliquots (20 μl) of a mixture containing doxorubicin, doxorubicinol, 7-hydroxydoxorubicin aglycone, and daunorubicin (1.0 μg ml^{-1}) were injected onto each system.

The response surface of the solvent selectivity triangle was calculated using the equation [12]:

$$R = B_1X_1 + B_2X_2 + B_3X_3 + B_4X_1X_3 + B_5X_1X_2 + B_6X_2X_3 + B_7X_1X_2X_3,$$

where $X_{1,2,3}$ refers to the position within the plane of the solvent selectivity triangle and B_1 to B_7 are the constants, calculated from the retention data as follows:

$$B_1 = E_1,\ B_2 = E_2,\ B_3 = E_3$$
$$B_4 = 4E_4 - 2(E_1 + E_3)$$
$$B_5 = 4E_5 - 2(E_1 + E_2)$$
$$B_6 = 4E_6 - 2(E_2 + E_3)$$
$$B_7 = 27E_7 - 12(E_4 + E_5 + E_6) + 3(E_1 + E_2 + E_3).$$

E_i refers to the retention time for each of the seven experimental points.

Factorial design. Eight mobile phases were prepared, incorporating 35% (v/v) acetonitrile and 0.05% (v/v) triethylamine, in a buffer containing 0.05 or 0.2 M Na_2HPO_4 adjusted to pH 3.2 or 5.2 with 0.025 or 0.1 M citric acid, either with or without 5% (v/v) THF.

The variables contributing the greatest individual effects and variable interactions were calculated using the generally accepted standard equations [12].

Modified simplex procedures. Thirteen mobile phases were prepared incorporating 35% (v/v) acetonitrile and 0.05% (v/v) triethylamine in buffers containing between 0.02 and 0.2 M Na_2HPO_4 adjusted prior to mixing to pH values in the range 3.2–5.2 with citric acid in the range 0.01–0.1 M. The chromatographic

response factor (CRF) was calculated using the equation:

$$CRF = \alpha' + \alpha'' + L,$$

where α' and α'' are the separation factors for epirubicinol and daunorubicin with their neighbouring peaks, and L is the total number of observed peaks.

Solid-phase extraction of anthracyclines

Stock solutions of doxorubicin, epirubicin, doxorubicinol, epirubicinol, 7-hydroxy- and 7-deoxydoxorubicin aglycones and 7-hydroxydoxorubicinol aglycone (10 μg ml^{-1}) and internal standard daunorubicin (20 μg ml^{-1}) were prepared in methanol. These were diluted to the required concentrations using 0.02 M NaH_2PO_4 (pH 4.0) immediately prior to use. Spiked horse serum samples were prepared containing 50, 200 and 500 ng ml^{-1} drug by addition of 0.1 ml of a mixture of doxorubicin, epirubicin and their metabolites at the appropriate concentration, and 0.1 ml daunorubicin (to give a final concentration of 200 ng ml^{-1}) to 0.8 ml horse serum. The spiked serum was diluted with 1 ml 0.02 M NaH_2PO_4 (pH 4.0) and mixed thoroughly. A 1 ml aliquot of this solution was added to a preconditioned Bond-Elut C_8 cartridge, and the column was washed with 3 × 1 ml water and 3 × 1 ml 0.02 M NaH_2PO_4 (pH 4.0). The adsorbed solutes were then slowly eluted from the column using 0.5 ml of 67.5% (v/v) acetonitrile and 0.05% (v/v) triethylamine in 0.2 M Na_2HPO_4 which was adjusted to pH 3.6 using 0.1 M citric acid. A 20 μl aliquot of extract was then directly injected onto a 250 × 4.6 mm i.d. Spherisorb ODS 1 analytical column. Recoveries were estimated using peak area ratios at three concentrations (50, 200 and 500 ng ml^{-1}). Extracted serum samples were compared with equivalent concentrations of standard drug solutions prepared in elution solvent, and peak area ratios calculated by comparison of drug with internal standard (daunorubicin).

Results and Discussion

Optimization of separation of anthracyclines by HPLC

Solvent selectivity triangle. This technique was used as a preliminary screening technique to select a possible region of the response surface in which to characterize the chromatographic performance. The most suitable type of organic modifier and the general composition of a suitable mobile phase were initially examined, based on the mobile phase composition described by Maesson [13]. Four compounds were used: a parent drug (doxorubicin), a polar metabolite (doxorubicinol), a relatively non-polar aglycone (7-hydroxydoxorubicin aglycone) and the internal standard (daunorubicin). Triethylamine (0.1%, v/v) was incorporated into all mobile phases to reduce chromatographic peak tailing by masking free silanol groups. Seven experiments were carried out with the different mobile phases (I–VII) and a region of mobile phase composition revealing the best relative retention for the worst separated peak pair in the initial chromatogram (Fig. 2) was identified.

These data were then used to calculate the response surface of the solvent selectivity triangle. The retention data for the seven experimental points are shown in Table 1. The resolution map obtained (Fig. 3) shows two areas of high peak resolution, one near the acetonitrile apex, and the other near the THF apex. However, it was found that a high proportion of THF in the mobile phase resulted in relatively long retention times (t_r = 33 min) with considerable peak broadening. Hence acetonitrile was shown to be the organic modifier of choice, although addition of <5% THF to the mobile phase resulted in improved peak separation.

Factorial design. Factorial design is used to elucidate the effect of multi-criteria response functions, through the relative importance and interaction of the experimental variables in the mobile phase composition [12]. In the procedure each selected parameter was investigated at two levels, assigning an upper (+) and lower (−) value. If n variables are investigated, then a total of 2^n experiments will be necessary to define the limits of the experimental domain or 'factor space'.

For this study, the resolution of epirubicinol and its metabolite 7-hydroxydoxorubicinol aglycone (the least resolved pair of chromatographic peaks) was examined using pH (3.2 and 5.2), buffer concentration (0.05 and 0.2 M Na_2HPO_4/0.025 and 0.1 M citric acid) and % (v/v) THF (0 and 5%) as the variables. The limits of these variables were selected using either data from the previous solvent selec-

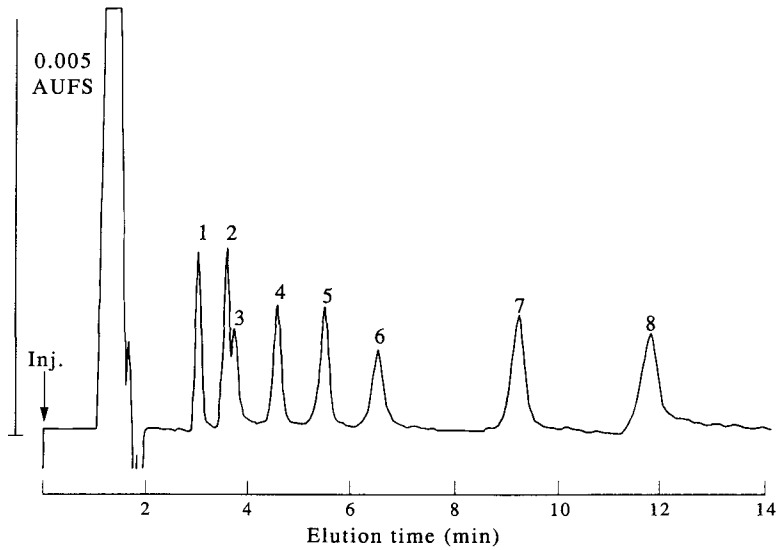


Figure 2
Initial HPLC chromatogram using acetonitrile–0.02 M sodium dihydrogen phosphate, containing 0.1% (v/v) triethylamine, adjusted to pH 4.0 using orthophosphoric acid (35:65, v/v) as mobile phase. Peaks: 1, doxorubicinol; 2, epirubicinol; 3, 7-hydroxydoxorubicinol; 4, doxorubicin; 5, epirubicin; 6, 7-hydroxydoxorubicin aglycone; 7, daunorubicin; 8, 7-deoxydoxorubicin aglycone.

Table 1
Retention data obtained from solvent selectivity experiments

Compound	Retention time (min)*						
	I	II	III	IV	V	VI	VII
Doxorubicinol	>40	2.9	8.1	>40	7.9	>40	11.2
Doxorubicin	>40	4.4	15.1	>40	9.6	>40	15.9
7-Hydroxydoxorubicin aglycone	>40	6.3	20.4	>40	17.3	>40	27.7
Daunorubicin	>40	8.8	32.9	>40	21.2	>40	28.9

*Numbers I–VII refer to mobile phases I–VII in the solvent selectivity triangle (see text).

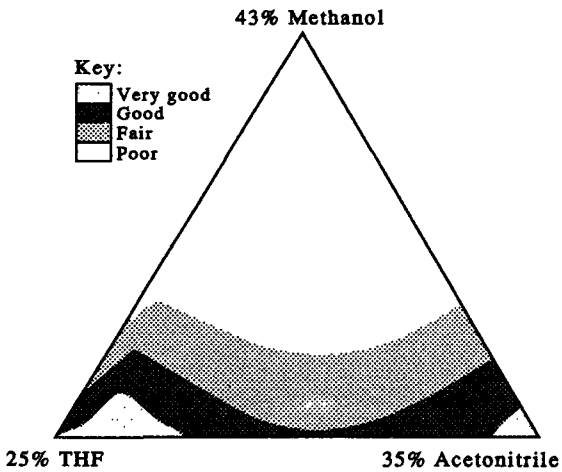


Figure 3
Resolution map for anthracycline separation from solvent selectivity triangle experiments.

tivity triangle experiments, or via additional preliminary experiments. The buffer chosen for these experiments was shown to provide adequate buffering capacity over the pH and concentration ranges selected. Aliquots of a mixture containing epirubicinol and 7-hydroxydoxorubicinol aglycone, doxorubicinol and doxorubicin (1.0 μg ml^{-1}) in mobile phase were injected onto the HPLC system described above, eluting with each of eight mobile phases as indicated in Table 2a. The results used to calculate the separation factor α (ratio of capacity factors for 7-hydroxydoxorubicinol aglycone and epirubicinol, respectively) are shown in Table 2b. These separation factors were then used to determine the variables with the greatest individual effects with the following results: pH (A) = 0.025; %THF (B) =

0.177; buffer concentration (C) = 0.566. The buffer concentration was found to be the variable with the greatest individual effect on solute separation in this system, followed by %THF and pH.

The calculated interactions were as follows: $A \times B = 0.021$; $B \times C = 0.006$; $A \times C = 0.037$ (where A, B and C are as given above). These data show that the greatest interaction occurs between A and C, namely pH and buffer concentration. Hence these parameters were used to define a two-dimensional factor space within which the final part of the optimization procedure could be performed.

Table 2a
Predetermined points used in the factorial design technique

Experiment	pH	%THF	Buffer conc. (M)*
1	3.2	0	0.05
2	3.2	0	0.20
3	3.2	5	0.05
4	3.2	5	0.20
5	5.2	0	0.05
6	5.2	0	0.20
7	5.2	5	0.05
8	5.2	5	0.20

*Buffer concentrations denote the Na_2HPO_4 component of the citrate buffer (e.g. 0.05 M refers to 0.05 M Na_2HPO_4–0.025 M citric acid).

Table 2b
Factorial design: chromatographic data for the worst separated pair of peaks

Experiment	t_1	t_2	t_0	k'_1	k'_2	α
1	5.37	5.61	1.66	2.23	2.38	1.07
2	3.64	4.92	1.71	1.13	1.88	1.66
3	5.47	5.47	1.64	2.34	2.34	1.00
4	3.58	4.76	1.65	1.17	1.88	1.61
5	5.32	4.99	1.53	2.48	2.26	1.10
6	3.88	5.41	1.51	1.57	2.58	1.64
7	5.63	5.28	1.62	2.47	2.26	1.09
8	4.07	5.62	1.52	1.67	2.68	1.60

t = Retention time (min); t_0 = time for solvent front; k' = capacity factor; α = separation factor (k'_2/k'_1), subscripts 1 and 2 refer to epirubicinol and 7-hydroxydoxorubicinol aglycone, respectively.

Modified simplex procedure. The previous experiments enabled an area of factor space to be defined for optimal response. Using acetonitrile as organic modifier (from the solvent selectivity triangle), the effect of altering pH and buffer concentration (the two most important variables as determined from the factorial design) was examined.

The basic simplex method uses fixed step movements of a geometric figure, the simplex (in this case for two variables, a triangle) across the response surface, with movements being determined by the results obtained after each step. The modified approach of Nelder and Mead was used in this study [14], in which the size of the simplex can be altered to more rapidly locate the optimum region. Ranking of responses was dependent on (a) the separation factor of any partially resolved peaks and (b) the number of chromatographic peaks observed and was reflected in the chromatographic response factor used. The experimental data is shown in Table 3 for the partially resolved peaks (7-hydroxydoxorubicinol aglycone, doxorubicin and 7-hydroxydoxorubicin aglycone, daunorubicin). From the calculated capacity factors (Table 3), the separation factors α_1 and α_2 were calculated.

Table 3
Chromatographic data for partially resolved peaks in modified simplex experiments

Experiment	t_0	t_1	t_2	t_3	t_4	t_i	k'_1	k'_2	k'_3	k'_4
1	1.22	4.21	4.45	7.48	7.89	13.41	2.46	2.66	5.15	5.49
2	1.23	5.38	5.74	9.43	9.43	17.76	3.38	3.66	6.67	6.67
3	1.11	4.61	5.04	8.78	8.78	15.92	3.15	3.53	6.90	6.90
4	1.13	4.70	4.70	8.25	8.96	15.13	3.15	3.15	6.27	6.90
5	1.21	4.80	4.93	7.85	8.31	15.43	2.95	3.06	5.47	5.85
6	1.12	5.25	5.25	9.31	10.41	17.54	3.67	3.67	7.28	8.26
7	1.14	5.31	5.85	9.17	11.86	17.10	3.64	4.12	7.02	9.37
8	1.14	4.44	4.69	7.45	8.96	13.36	2.90	3.11	5.53	6.86
9	1.11	9.68	9.68	12.16	20.85	20.85	7.75	7.75	9.99	17.80
10	1.16	6.01	6.19	10.54	12.74	20.08	4.18	4.33	8.08	9.97
11	1.16	5.42	5.65	9.40	11.45	17.58	3.68	3.88	7.11	8.88
12	1.16	6.99	7.33	11.78	12.41	24.13	5.01	5.31	9.13	9.67
13	1.10	5.00	5.46	14.04	14.04	15.46	3.52	3.94	11.70	11.70

t = retention time (min); t_0 = time (min) for solvent front; t_i = time (min) of last eluting peak. Key: 1, 7-hydroxydoxorubicinol aglycone; 2, doxorubicin; 3, 7-hydroxydoxorubicin aglycone; 4, daunorubicin. $\alpha_1 = k'_2/k'_1$; $\alpha_2 = k'_4/k'_3$.

Intially, three widely spaced points were chosen to identify the most favourable region of the response surface. As the experiments progressed, the simplex was forced away from regions of poor response towards an area of optimal response. Rapid location of the suspected global optimum was achieved (point 7 in Fig. 4), leading to a final mobile phase composition of acetonitrile–0.06 M Na_2HPO_4 containing 0.05% (v/v) triethylamine adjusted to pH 4.6 with 0.03 M citric acid prior to mixing (35:65, v/v). A further six experiments confirmed the area of optimal response. The final chromatogram (Fig. 5) shows baseline separation of all eight peaks within 13.7 min

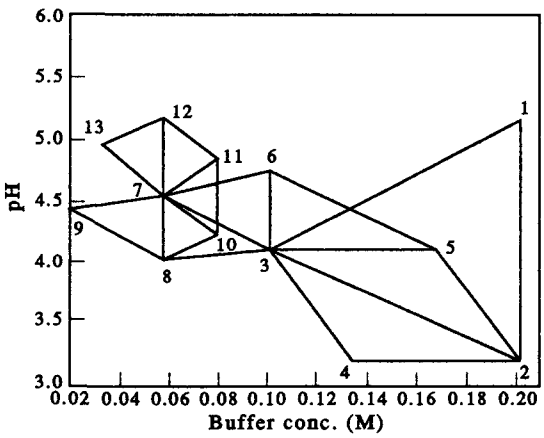


Figure 4
Modified simplex diagram showing area of optimal anthracycline separation (point number 7).

using a 150 × 4.6 mm i.d. column. In this case, the modified simplex procedure was able to rapidly locate the position of optimal response within a defined factor space.

Solid-phase extraction

To facilitate a simple and efficient method of extraction of anthracyclines from biological matrices, a solid-phase extraction procedure using a C_8 sorbent was developed. The C_8 sorbent enabled all the anthracyclines to be extracted on the SPE column from biological matrices by means of both polar and non-polar interactions. The anthracyclines were extracted from spiked horse serum using Bond-Elut reversed-phase cartridges. An elution solvent was developed with both high organic modifier content and high buffer ionic strength, and contained acetonitrile–0.2 M Na_2HPO_4 containing 0.05% (v/v) triethylamine, adjusted to pH 3.6 using 0.1 M citric acid (67.5:32.5, v/v).

Fluorescence detection was used to enhance the sensitivity and selectivity of solute detection in all SPE studies. Extracted blank serum produced a chromatogram with no interfering peaks. The results of this work (Table 4 and Fig. 6) show a high and reproducible recovery for doxorubicin (200 ng ml^{-1}) 94 ± 8% and epirubicin 96 ± 8% (n = 5), with higher per cent recoveries for all other anthracyclines/anthracyclinones. The data for

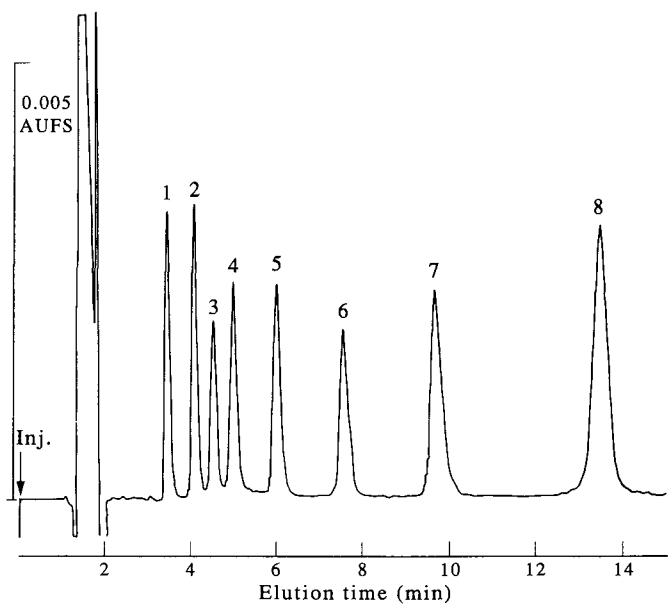


Figure 5
HPLC Chromatogram, using 150 × 4.6 mm i.d. Spherisorb ODS 1 column eluting with acetonitrile–0.06 M Na_2HPO_4 containing 0.05% (v/v) triethylamine, adjusted to pH 4.6 with 0.03 M citric acid prior to mixing (35:65, v/v). Peaks as for Fig. 2.

Table 4
Anthracycline recoveries using solid-phase extraction from spiked horse serum (mean ± RSD, $n = 5$)

Compound	Initial drug concentration in serum (ng ml^{-1}) 50	200	500
Doxorubicin	88.3 ± 4.6	94.2 ± 8.4	93.9 ± 9.2
Epirubicin	97.4 ± 5.3	96.1 ± 7.7	94.0 ± 8.9
Doxorubicinol	92.3 ± 5.0	94.4 ± 8.0	93.5 ± 9.0
Epirubicinol	91.1 ± 8.3	95.7 ± 7.7	93.6 ± 9.5
7-Hydroxydoxorubicin aglycone	98.2 ± 4.2	98.7 ± 7.1	97.7 ± 9.4
7-Hydroxydoxorubicinol aglycone	94.0 ± 3.5	98.4 ± 6.5	94.8 ± 7.8
7-Deoxydoxorubicin aglycone	95.8 ± 4.2	98.5 ± 9.3	92.6 ± 5.8

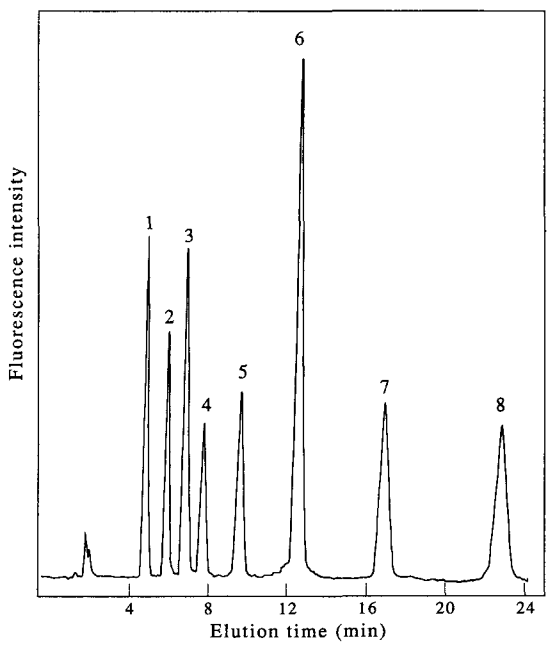


Figure 6
HPLC Chromatogram of extracted serum samples, using 250 × 4.6 mm i.d. Spherisorb ODS 1 column eluting with mobile phase as for Fig. 5. Peaks as for Fig. 2.

lower drug concentrations (50 ng ml^{-1}) showed good recovery for epirubicin 95 ± 5%, but lower recovery for doxorubicin 88 ± 4.5%, with all other anthracyclines/anthracyclinones extracted with >90% efficiency. These data compare favourably with literature methods describing solid-phase extraction of doxorubicin, epirubicin and their metabolites. The methods for extraction of doxorubicin, or doxorubicin and doxorubicinol which utilized C_2 [15] or C_{18} extraction columns [16] cite recoveries up to 92%. The literature methods describing solid-phase extraction of doxorubicin and epirubicin metabolites generally give recoveries <80% [13, 17]. The lower detection limit of the assay for both doxorubicin and epirubicin in serum was 1 ng ml^{-1} (at a signal to noise ratio of 3), with a limit of quantitation of 10 ng ml^{-1}. The methodology allows serum concentrations <10 ng ml^{-1} to be quantitated by pre-concentration on the SPE cartridge.

These data show that biological matrices containing doxorubicin, epirubicin and their clinically important metabolites can be rapidly and selectively extracted from carrier tissues and fluid by means of a facile system using Bond-Elut cartridges. The adsorbed drugs can be rinsed with buffer to remove extraneous materials, then eluted from the sorbent and directly injected onto a HPLC system. This method avoids the necessity for solvent extraction of anthracyclines from biological tissues and fluids, which is problematic owing to major differences in the lipid solubilities of these drugs, ranging from the relatively lipophilic 7-deoxyaglycone, to the amphiphilic parent drugs and their 13-*S*-dihydrometabolites. However the solid-phase extraction system described above enables good recovery of all anthracyclines and their metabolites from biological samples despite their disparate physicochemical properties.

Linearity and precision

To determine linearity of the assay, aqueous solutions of each drug were prepared in the range 50–1000 ng ml^{-1}. Linear regression analyses of the peak area ratios obtained from chromatograms from 10 injections at each of six different drug concentrations gave correlation coefficients >0.998 in the range 50–800 ng ml^{-1} for all components except 7-deoxydoxorubicin aglycone, where $r = 0.992$. Deviation from linearity was observed for all aqueous solutions of anthracyclines at concentrations >800 ng ml^{-1}, probably due to self-association of the molecules at higher concentrations.

Table 5
Calibration data for regression analysis of anthracyclines and anthracyclinones in spiked horse serum

Compound	Slope*	Intercept	Correlation coefficient
Doxorubicin	0.65 ± 0.01	−0.17	0.9976
Epirubicin	1.03 ± 0.02	−0.25	0.9984
Doxorubicinol	1.25 ± 0.02	−0.33	0.9968
Epirubicinol	0.99 ± 0.02	−0.27	0.9972
7-Hydroxydoxorubicinol aglycone	1.70 ± 0.05	−0.13	0.9950
7-Hydroxydoxorubicin algycone	2.76 ± 0.05	−0.69	0.9961
7-Deoxydoxorubicin aglycone	2.49 ± 0.04	−0.36	0.9975

*Each calibration graph represents four concentrations in the range 50–500 ng ml^{-1}, with five replicates at each point.

Calibration curves for all solutes in extracted serum samples were established in the clinically relevant range of 50–500 ng ml^{-1}. Five samples at each of four different concentrations gave linear plots for all components (Table 5) with correlation coefficients >0.995; differences in the slopes of the calibration lines reflect differences either in recovery, or in the fluorescence quantum yields for the solutes. The within-day precision of the assay was established by the analysis of five extracted serum samples spiked with the analytes at three different concentrations (Table 4). Relative standard deviation (RSD) values varied between 3.5 and 9.5% and were similar to those described elsewhere [13].

Conclusions

This study has successfully demonstrated the utility of formal optimization techniques in the HPLC analysis of anthracyclines and their metabolites. The separation of eight components using a 150 mm column was achieved in a run time of 13.7 min, only 2 min longer than the initial chromatogram, where only partial component resolution was observed. For the 250 mm column all solutes were resolved in 24 min. The most important mobile phase variables in anthracycline HPLC analysis were determined to be pH and buffer concentration, and application of formal optimization procedures provided data which determined the optimal mobile phase composition giving baseline resolution of eight anthracycline/anthracyclinone solutes; this comprised acetonitrile–0.06 M Na_2HPO_4, containing 0.05% (v/v) triethylamine, adjusted to pH 4.6 with 0.03 M citric acid (35:65, v/v).

It has also been shown that a rapid and highly selective solid-phase extraction of doxorubicin, epirubicin and their clinically important metabolites can be achieved, using C_8 Bond-Elut cartridges followed by HPLC analysis. The described methodology yielded high drug recoveries and reproducibilities across the spectrum of physicochemical properties.

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