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The effect of surfactants on the high-performance liquid chromatography of anthracyclines

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Abstract: A rapid, reversed-phase high-performance liquid chromatographic method is described for the determination of the antineoplastic drug, doxorubicin (Adriamycin), and its major metabolites in plasma. The addition of anionic, cationic or non-ionic surfactants was found to reduce k'; the mechanisms of this finding are discussed and the performance of an optimized method using a non-ionic surfactant, Brij-35, is described for plasma samples.

Keywords: *Reversed-phase high-performance liquid chromatography; anthracyclines; surfactant effects; fluorescence detection; doxorubicin; Adriamycin.*

Introduction

The antineoplastic drug, doxorubicin (Adriamycin, ADR) is widely used in conjunction with other drugs for the treatment of a variety of neoplasms. The use of the drug is associated with a dose-related cardiomyopathy. It has been suggested recently that toxicity may be reduced or eliminated if the plasma concentrations, which reflect tissue concentrations, are kept below a critical level but this has yet to be proven.

ADR is converted *in vivo* to the pharmacologically active metabolite doxorubicinol (ADR-ol) [1], which is also cardiotoxic, and to doxorubicinone (ADR-one), an aglycone, the toxicity of which is unknown (Fig. 1). A variety of conjugates, sulphates and glucuronides have also been identified in urine [2, 3]. In the absence of renal failure, only ADR, ADR-ol and ADR-one have so far been found in plasma.

Although methods for the determination of ADR have been available for some years, using RIA [4], or fluorescence [5], it is only with the advent of high-performance liquid chromatography (HPLC) that selective methods have become available, by which both the parent molecule and its metabolites can be determined. HPLC methods have been reported with detection in the visible range [6–8] and in the ultraviolet range [9] as well as detection by fluorescence [10–14]. Reported reversed-phase systems showed poor efficiencies in the authors' laboratories and even the use of an ion-pair reversed-phase system [8] did not give a reproducible performance for biological specimens. The aim of the present work was to optimize an ion-pair reversed phase system for clinical studies.

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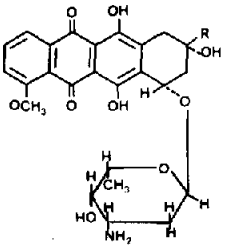

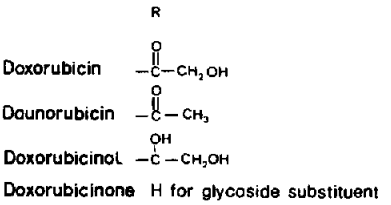

Figure 1
The structure of doxorubicin (ADR), daunorubicin (DAUN), doxorubicinol (ADR-ol) and doxorubicinone (ADR-one).

Materials and Methods

Reagents

Doxorubicin (ADR), doxorubicinol (ADR-ol), doxorubicinone (ADR-one) and daunorubicin (DAUN) were donated by Farmitalia (Milan, Italy).

Sulphonic acids (HPLC grade) and quaternary ammonium salts (HPLC grade) were obtained from Fisons (Loughborough, UK).

All solvents were obtained from Rathburn Chemicals (Walkerburn, Peebleshire, UK) and were HPLC grade.

Brij-35 (polyoxyethylene lauryl ether) was obtained from Technicon (Basingstoke, Hants, UK).

Apparatus

An HPLC system consisting of an LC-XPS single-piston reciprocating pump with an LC-FL fluorescence detector fitted with interference filters at 450 nm (excitation) and 550 nm (emission) (Pye Unicam, Cambridge, UK) was used with a PM 8251 single-pen chart recorder (Pye Unicam).

The column was a 100 × 5 mm i.d. stainless steel column (Shandon Southern, Runcorn, Cheshire, UK), slurry packed in the laboratory with 5-μm Hypersil ODS (Shandon Southern, Runcorn, Cheshire, UK). Injections were made via a Rheodyne 7125 valve fitted with a 20-μl loop.

Extraction procedure

The procedure used for the extraction of plasma samples was similar to that of Shinozawa [15] and was found to be satisfactory.

To 1 ml of plasma was added 100 μl of internal standard solution (200 ng DAUN in dichloromethane). The plasma was mixed with 1 ml of 10 mM phosphate-buffered saline (pH 7.8), and extracted with 10 ml of dichloromethane–isopropanol (4:1, v/v) for 10 min.

After centrifugation the upper aqueous layer was aspirated and the organic layer transferred to a conical glass tube and evaporated to dryness at 50°C under a stream of nitrogen. The residue was dissolved in 100 μl of the HPLC eluant and mixed with a vortex mixer; 20 μl was injected on the column.

Study of chromatographic conditions

In order to study the effect of the addition of different surfactants to the eluting solvent, 20 μl of a standard solution containing 20 ng each of ADR, ADR-ol, ADR-one and DAUN in methanol was injected on the column. The initial chromatographic eluant consisted of acetonitrile–0.01 M phosphoric acid (35:65, v/v).

Modification of the eluant solution

To the eluant solution were added a series of surfactants as follows:

Anions: pentane, hexane, heptane and octane sulphonic acids at final concentrations of 0.75–6 mM; the ionic strength was kept constant by the addition of appropriate amounts of sodium sulphate (original eluant μ = 0.06 M; surfactant modified eluant μ_{tot} = 0.078 M; thus $\Delta\mu$ = 0.018 M).

Cations: Dodecyltrimethylammonium bromide or tetra-ethylammonium bromide at concentrations of 0.75–6 mM.

Non-ionic surfactant: Brij-35 at concentrations of 0.75–6 mM.

The column was equilibrated for several hours with each eluant and injections of the analyte mixture were made until the k' values of the solutes were reproducible. Between each experiment surfactants were removed from the column by washing with acetone overnight.

Investigation of the performance of the extraction/optimized assay

After the chromatography had been optimized as described above, the efficiency of the complete assay was assessed using spiked plasma samples.

Results

The effect of the addition of a variety of surfactants

Separation of the analytes of interest using the original solvent is shown in Fig. 2; however, ADR-one could not be resolved from a peak derived from the biological matrix, that was eluted early. The peak obtained from DAUN had a k' of 11 and tailed. At low pH the amino-group of the glycoside would be protonated and thus would be amenable to the formation of ion pairs with anionic surfactants, which should improve the peak shape [16].

Sodium sulphate alone caused a reduction in analyte k' of approximately 10%. The results obtained with the sulphonic acid eluants at constant ionic strength are shown in Table 1. ADR, ADR-ol and DAUN showed an initial reduction in k' but as increasing surfactant was added the retention increased; the extent of this increase was related to alkyl chain length. ADR-one, however, showed no change in k' with any anionic surfactant, resulting in crossover with ADR-ol. With lower surfactant concentrations the k' of ADR was decreased so that this substance was poorly resolved from ADR-one. The best resolution was given with a heptane sulphonic acid concentration of 4.5 mM or greater; however, although acceptable efficiency was achieved, the separation factor was too small ($Rs < 0.9$) between ADR-one and ADR-ol and between ADR-one and ADR.

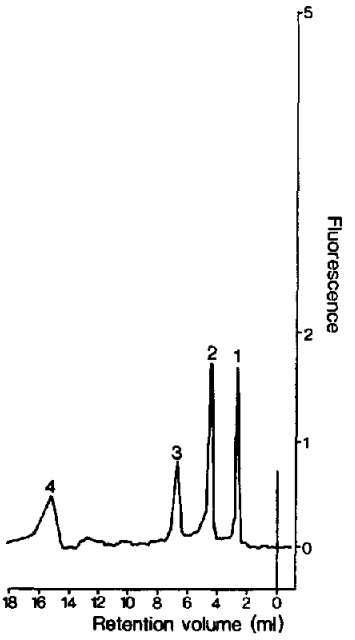

Figure 2
Chromatogram of 20 ng each of doxorubicin and metabolites in methanol. Mobile phase acetonitrile–10 mM phosphoric acid (35:65, v/v). Flow rate, 1 ml min^{-1}. Peaks identities: 1 = ADR-one, 2 = ADR-ol, 3 = ADR, 4 = DAUN (internal standard).

Table 1
Effects of anionic and cationic surfactants on the k' of ADR-one, ADR-ol, ADR and DAUN

Surfactant concentration (mM)	ADR-one	ADR-ol	ADR	DAUN
0	1.8	2.5	4.5	11
Octane sulphonic acid				
0.75	1.8	1.2	1.8	3.4
1.5	2.0	1.4	2.2	4.7
3.0	1.9	1.5	2.3	5.3
4.5	2.0	1.8	2.6	6.2
6.0	2.0	1.8	2.7	6.2
Heptane sulphonic acid				
6.0	1.8	1.0	2.4	5.8
Hexane sulphonic acid				
6.0	1.8	1.0	2.0	5.0
Pentane sulphonic acid				
6.0	1.8	1.0	1.9	4.9
DDTAB*				
6.0	1.8	0.2	0.2	0.2
TEAB†				
6.0	1.8	1.0	1.2	2.5

Ionic strength maintained constant using sodium sulphate (Δu = 0.018 M). Data for concentrations between 0 and 6 mM omitted for surfactants other than octane sulphonic acid.

*DDTAB = dodecyltrimethylammonium bromide.

†TEAB = tetra-ethylammonium bromide.

The effect of two cationic surfactants, dodecyltrimethylammonium bromide and tetraethylammonium bromide, was investigated over the concentration range 0.75–6 mM. The k' for these four analytes are given in Table 1. There was a dramatic reduction in k' for ADR, ADR-ol and DAUN with ADR-one again unaffected. The net effect of the use of cationic surfactants was to give very rapid elution of all four analytes but with loss of resolution ($Rs < 0.5$) between ADR-one, ADR-ol and ADR. The effect of tetraethylammonium bromide was less pronounced than that of dodecyltrimethylammonium bromide although ADR-one was eluted after both ADR and ADR-ol when the surfactant concentration exceeded 3 mM.

The effect of the non-ionic surfactant Brij-35 on the separation is shown in Table 2. Again there is no effect on the k' of ADR-one; reduction in the k' of ADR, ADR-ol and DAUN was proportional to the concentration of Brij-35. The most satisfactory resolution, with a reduction in k' (from 11 to 7.7) for DAUN at 6 mM Brij-35, is shown in Fig. 3. The resolution between ADR-one and ADR-ol was good ($Rs = 2.0$) as was that for ADR-one and ADR ($Rs = 3.6$). The number of theoretical plates calculated for ADR under these conditions was 4700.

The total time necessary for a complete chromatogram using this system could be reduced to 8 min with a flow rate of 1.5 ml min^{-1}. This was the method of choice.

Table 2
Effect of a non-ionic surfactant on k' of ADR-one, ADR-ol ADR and DAUN

Surfactant concentration (mM)	k'			
Non-ionic surfactant Brij-35	ADR-one	ADR-ol	ADR	DAUN
0	1.8	2.5	4.5	11.0
0.75	1.8	2.4	4.2	10.0
1.5	1.8	2.3	4.1	9.5
3.0	1.8	2.2	4.0	8.8
4.5	1.8	2.1	3.9	8.0
6.0	1.8	2.0	3.8	7.7

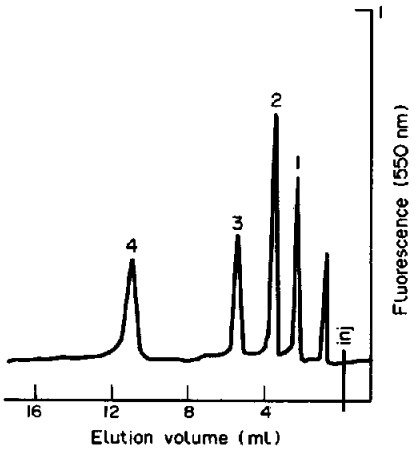

Figure 3
Chromatogram of ADR and metabolites, after extraction from plasma, mobile phase as in Fig. 2 but with 6 mM Brij. Flow rate 1 ml min^{-1}. Peak identities as in Fig. 2. The un-numbered peak is a plasma component.

Performance characteristics of the method

The performance of the combined extraction and chromatography was assessed using spiked plasma samples. Recovery, assessed by comparison of peak areas with those obtained from a directly injected standard mixture of ADR, ADR-ol and ADR-one, was 95.4 ± 3.9%, 92.2 ± 4.7% and 82.1 ± 5.1%, respectively at a concentration of 100 μg l^{-1}. Precision was 4.1%, 5.1% and 6.3% for ADR, ADR-ol and ADR-one at 100 μg l^{-1} and 3.6%, 4.8% and 5.5%, respectively, at 500 μg l^{-1} (n=20). Sensitivity (peak height equivalent to 2× baseline noise in repeated experiments) was 1.5 μg l^{-1} (3 nM). Specificity: the following drugs, all of which were found to be co-prescribed to at least one patient, were found to give no interfering peaks at concentrations of 1 g l^{-1}: cyclophosphamide, dacarbazone, 5-fluorouracil, vincristine, vinblastine, vindecine, *cis*-platinum, cyta-arabine, methotrexate and its metabolites, trimethoprim, paracetamol and diazepam.

Overall performance

One example of the chromatogram obtained from a patient not receiving cytotoxic therapy is shown in Fig. 4a; the chromatogram obtained from the plasma of a patient 3 h after a dose of ADR (30 mg m^{-2}) is shown in Fig. 4b. ADR and ADR-ol are clearly separated from the non-retained peak found in plasma extracts in a total run time of 8 min at a flow rate of 1.5 ml min^{-1}. Plasma concentrations of ADR, ADR-ol and ADR-one during and after an i.v. infusion of 10 mg h^{-1} in one patient are shown in Fig. 5.

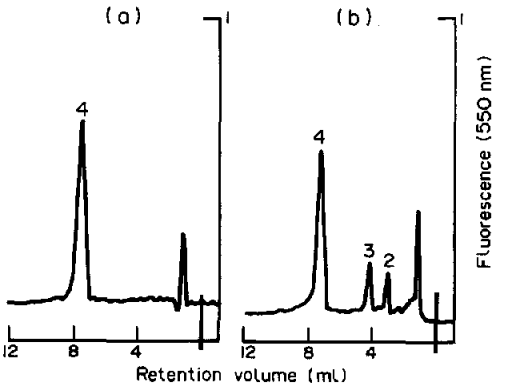

Figure 4
(a) Chromatogram of plasma extract from a drug-free subject. (b) Chromatogram of plasma extract from a patient treated with 30 mg m^{-2} ADR. Conditions as in Fig. 3 except for flow rate 1.5 ml min^{-1}.

Discussion

Reversed phase methods for the separation of anthracyclines have tended towards a common norm employing either acetonitrile or methanol and an acidic aqueous phase, usually phosphoric acid at a pH of approximately 2.2. At this pH the glycosidic amine present in the parent drug and oxidized metabolites is assumed to be protonated; separation by a conventional reversed phase mechanism with predictable effects using ion-pairing agents were expected and the intention of the authors was merely to optimize a previously published method.

The addition of cationic surfactants, tetra-ethylammonium bromide and dodecyltrimethyl ammonium bromide produced the expected reduction in k' for the glycoside-containing molecules consistent with counter-ion displacement. When anionic surfac-

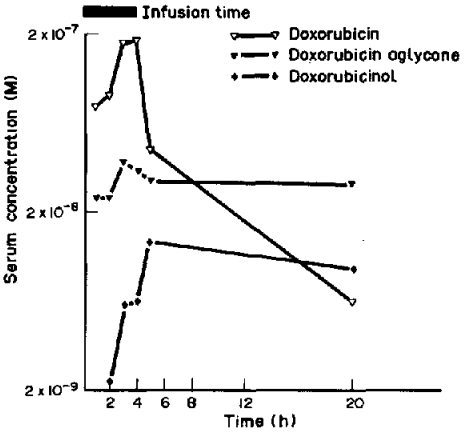

Figure 5
Plasma concentrations of ADR, the ADR-one and ADR-ol during and after the infusion of ADR over 4 h. Conditions as for Fig. 4b.

tants were used a decrease in k' was again obtained; this is contrary to theory and indicated that the mechanism is not the classical mechanism of reversed-phase ion-pair formation. Similar behaviour has been noted in a reversed-phase system with sodium dodecyl sulphate [8] but only for eluants of low water content. There are several possible explanations for this finding. Van Lanker *et al.* [8], using the longer chain sodium dodecyl sulphate as an ion-pairing agent, found the expected increase in k' with increasing concentration of sodium dodecyl sulphate up to a critical concentration of 30 mM; further increases in anionic surfactant concentration gave rise to a decreased k'. The explanation by those authors was the formation of micelles in the eluant. Dielder and van den Berg [17] had also proposed micelle formation at high concentrations of anionic ion-pairing agents; they calculated that for octane sulphonic acid, micelle formation occurred at concentrations in excess of 100 mM. However attractive this explanation may be, the concentrations used in the present experiments, with a maximum of 6 mM, did not approach those used by Dielder and van den Berg [17]; micelle formation is an unlikely mechanism for the observed reduction in k' in the present work.

Bij *et al.* [18] showed that analytes containing ionized amine groups at low pH interact readily with residual silanol groups on reversed-phase packing materials. Although the Hypersil ODS used in the present experiments is a well end-capped material and the results were reproducible on freshly prepared columns, there is still likely to be a significant proportion of silanol groups available for interaction with cationic solutes; indeed it is the presence of such silanol groups which may provide an element of the selectivity observed in so-called reversed-phase separations of these analytes.

The explanation proposed [18] for a reduction in the k' of cations with the addition of an ionic surfactant is that the affinity of the anionic pairing ion in the mobile phase exceeds that of the silanol groups thus leading to ion-pair formation in the eluant and decreased retention. This explanation, however, requires that the proposed mechanism swamps the expected reversed-phase behaviour. The present authors accept that it may play some part in providing selectivity but it cannot be invoked as the sole explanation for the observed behaviour. As sodium sulphate was used to maintain ionic strength, changes in this parameter were not responsible for the variations noted.

Results of experiments in which the non-ionic surfactant Brij-35 was added to the eluant showed a modest decrease in k' for all eluants with some improvement in

resolution. Ghaemi and Wall [16] showed that silica (normal phase) loaded with a similar non-ionic surfactant, TWEEN-80, behaved as a reversed-phase material because of masking of the silanol groups. Such a dynamic reversed phase would be more polar than that of the octadecyl groups surrounding it. The overall effect would be one of increased polarity of the stationary phase, which would lead to a reduction in k' in a reversed-phase ion-pair separation.

Ionic surfactants may adsorb on a reversed-phase silica material in one of two ways; the surfactant is usually thought to be bound by hydrophobic linkages to the octadecyl groups. In this situation, the exposed charged portion of the molecule will attract solute ions of the opposite charge, resulting in a layer with the characteristics of a dynamic ion-exchanger. This layer will interact with adjacent uncapped silanol groups [18]. However, this mechanism was proposed for cationic ion-pair agents and does not explain the present findings. The binding of a charged surfactant to the reversed-phase material does, however, lead to a bound layer of increased polarity due to the negative charge. Increasing concentrations of surface-bound surfactant will increase the polarity of the surface layer leading to reduced avidity for the relatively non-polar analytes and reduction in k'. This explanation is both consistent with the findings and, as expected, the observed effect is greater for the more polar charged surfactants than for the uncharged Brij-35.

It is postulated therefore that the observed effects are due to relative changes in the polarity of the reversed-phase surfaces coupled with capping of the residual silanol groups, giving a more homogenous surface with high polarity and some cation-exchange characteristics.

The selectivity of the method is comparable with the results quoted by others [11, 12, 16]. For anionic surfactants the observed k' is proportional to chain length, the cations being less retained in the presence of the shorter, more polar anions. This effect has been noted previously by other workers [11, 16, 19] and may be due to increased inherent acidity or steric hindrance.

The final assay employs Brij-35 as the organic modifier. This method has proved robust in the authors' hands, giving an acceptable k' for all four analytes and allowing predictable 'tuning' of the method without the risks of co-elution and crossover observed when anionic surfactants are used. The efficiency of the assay is superior to that quoted by van Lanker *et al.* [8] and the assay has been found to be transferable to other groups of workers for use in the routine measurement of adriamycin and its major metabolites in plasma; preliminary results indicate that the assay is also applicable to urine.

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