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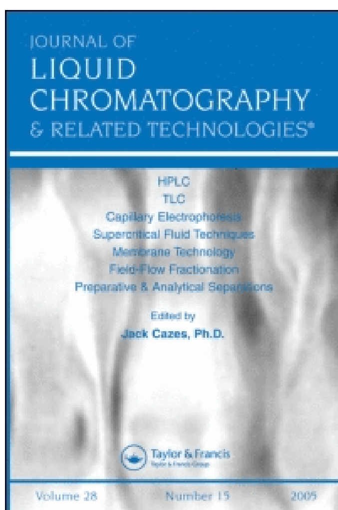


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# UTILITY OF ION-PAIR CHROMATOGRAPHY FOR ANALYSIS OF SOME ANTHRACYCLINES IN PLASMA AND URINE

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## ABSTRACT

A high performance liquid chromatographic assay was developed and validated for a simultaneous determination of adriamycin and its metabolites adriamycinol and adriamycinone as well as daunomycin in plasma. Acetonitrile and trichloroacetic acid were employed in removing proteins and extracting the drugs and metabolites into the supernatant for HPLC. Enhancement of chemical stability of the anthracyclines during sampling procedure was investigated using 0.01 M γ-cyclodextrin. The processed samples were chromatographed using an ODS/TM column (150 X 4.6 mm I.D.) eluted with a mobile phase composed of 35% acetonitrile and 65% (0.1M) monobasic phosphate, containing 0.3% heptafluorobutyric acid, pH 3.



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The detection was performed fluorimetrically at emission and excitation wavelengths of 555 and 460 nm, respectively. The described method could be directly applied for analysis of tested anthracyclines in human urine without sample pretreatment.

## INTRODUCTION

Anthracyclines belong to a group of structurally related antibiotics used as antineoplastic agents. Adriamycin (ADR) and daunomycin (DAU) are the best known of several hundreds anthracyclines. Daunomycin has been used primarily in acute leukemia whereas ADR has a broad spectrum activity against a variety of malignant diseases, including leukemia, lymphoma, lung cancer and also against a number of solid tumors. The use of both drugs is associated with a dose-related cardiomyopathy which accompanies long-term treatment.[1] Adriamycin is converted in vivo to the pharmacologically active metabolites adriamycinol (ADR-OL) which is cardiotoxic and adriamycinone (ADR-ONE).[2] Therefore, monitoring plasma levels of ADR, and its metabolites, seems to be necessary in order to provide information that could help our understanding of variations in clinical response and toxicity.

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Several analytical methods have been described to determine the concentration of these compounds and their metabolites in biological fluids and tissues. These include TLC[3] immunoassay[4] and HPLC.[5-8] Biological samples must be treated prior to chromatography to remove constituents whose peaks would interfere with that of anthracyclines and constituents that shorten column life time. Traditional solvent extraction is tedious due to emulsion formation, especially at liquid-liquid interface[9-11] while solid-phase extraction is time consuming.[12,13] Even by protein precipitation, the extraction is sometimes rarely quantitative.[14,15] This can be explained by the lower extraction coefficient or by the lower stability during sampling procedure.

Cyclodextrins (CDs) are capable of forming inclusion complexes with a variety of hydrophobic molecules by taking up a whole molecule, or some part of it, into the cavity. An inclusion complex formation of drug with CDs is known to bring about an enhancement of chemical stability.[16] The stability of the formed complex depends on how well the guest molecule fits into the cavity and the strength of the most hydrophobic interaction between the guest and host molecules.

In the present work an improved protein precipitation technique in combination with ion-pair (IP) chromatography was developed for the separation and quantitation of ADR and its two main metabolites, ADR-OL and ADR-ONE as well as DAU in human plasma. The influence of K-CD as stabilizing agent during

sampling procedure was achieved in this work. The described procedure was applied directly for analysis of the tested compounds in human urine without sample pretreatment.

## MATERIALS AND METHODS

### Materials

Adriamycine hydrochloride and daunomycin hydrochloride were obtained from Kyowa Hakki Co.,Tokyo, Japan). Adriamycinol and adriamycinone were kindly supplied by Farmitalia (Milan, Italy) and used without further purification. Sodium heptane-1-sulfonate (SHS), heptafluorobutyric acid (HFBA) and trichloroacetic acid (Nacalai Tsque Co., Kyoto, Japan) were chemically pure grade. Acetonitrile was HPLC grade (Kanto Chemical Co., Tokyo, Japan). TSK gel ODS/TM silica was obtained from (Tosoh Co., Tokyo, Japan). All other chemicals used were analytical grade.

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### Instruments

An HPLC apparatus consisted of a model HLC-803 pump (Toyo, Soda, Japan), equipped with a Rheodyne model 7125 syringe loading sample injector with 10 or 100 μL loop (Rheodyne, Berkeley, CA, USA). This system was equipped with a stainless-steel analytical column (150 X 4.6 mm i.d.) slurry-packed with ODS/TM silica (pore size 80Å, particle size 5μm ). The chromatographic detector used was a Shimadzu RF-530 fluorescence monitor (Shimadzu, Kyoto, Japan).

### Standard Solutions

A stock solution of ADR, ADR-OL, ADR-ONE and DAU (500 nmol/mL) was prepared by dissolving an accurately weighed amount of the drugs and metabolites in 25 mL distilled water. Aqueous calibration solutions of tested compounds were prepared by dilution of stock solution to give a final concentration range of 25-250 nmol/mL. Spiked plasma and urine samples for calibration curves were prepared daily by diluting the appropriate volume of standard solutions of ADR, ADR-OL, ADR-ONE and DAU with drug-free human plasma or urine in a ratio of 1 : 9 to produce a concentration range of 2.5-25 nmol/mL. The spiked plasma in the final dilution contains 0.01 M CD. Spiked plasma and urine samples were stored at -20 °C until use.

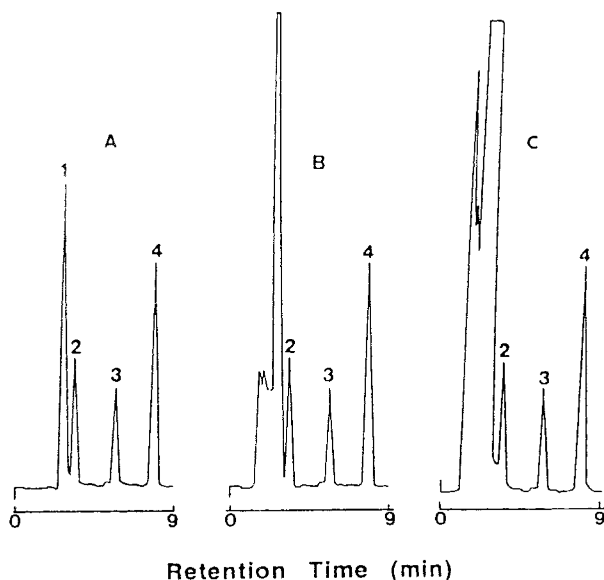


**Figure 1**. Chromatographic profiles of (A) standard ADR (2), ADR-OL (1), ADR-One (4), DAU (3); (B) drug-free human plasma spiked with 20 nmol/mL tested anthracyclines; (C) drug-free human urine spiked wiht 20 nmol/mL tested anthracyclines.

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## Chromatographic Conditions

Isocratic elution mode was employed for resolution and quantitation of ADR, ADR-OL, ADR-ONE and DAU. The mobile phase consisted of acetonitrile and 0.1 M monobasic phosphate containing 0.3% HFBA (35-65). Ion-pair reagent was added to the phosphate solution and pH was adjusted to 3 by addition of sodium hydroxide, then filtered before the required volume of acetonitrile was added. The mobile phase was degassed before use. The flow rate was 1 mL/min and the column temperature was ambient. The column effluent was monitored fluorometrically at excitation and emission wavelengths of 460 and 555 nm, respectively. At the end of analysis, the analytical column was thoroughly rinsed by methanol and then by 50% methanol in water.

## Sample Preparation

Into a glass centrifuge tube, acetonitrile (150 μL) was added to the spiked plasma (150 μL, 10 nmol/mL) and then vortex-mixed (10 sec). The precipitated

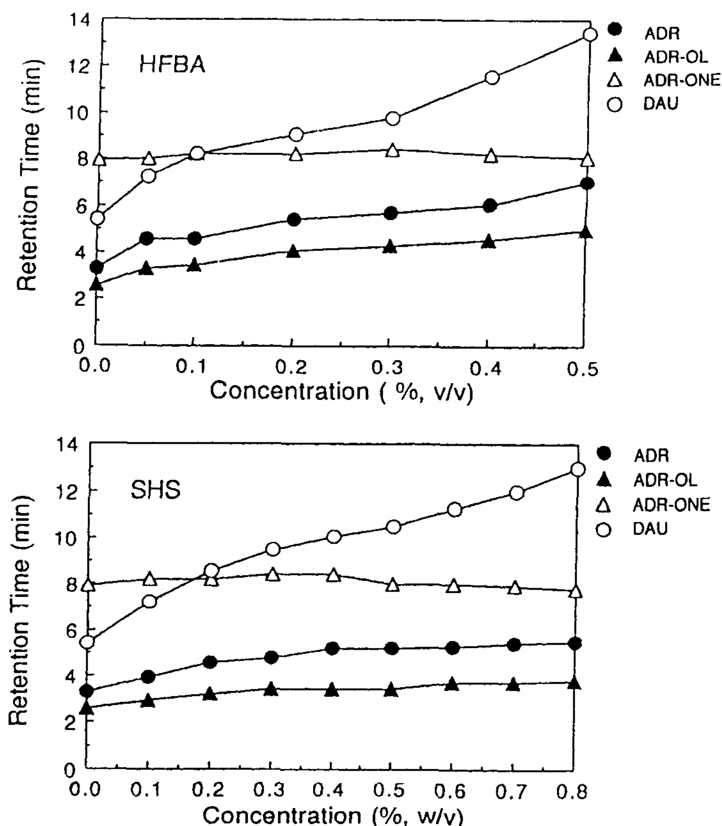


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**Figure 2**. Influence of pairing ion concentration on the retention time of tested anthracyclines.

proteins were separated by centrifugation (5 min, 3000 rpm). From acetonitrile layer, a volume of 200 μL was transferred to another tube and evaporated till dryness under vacuum. The residue was reconstituted in 100 μl 0.1 M monobasic phosphate, pH 3.

An aliquot of 10 μL was injected onto the column and peak heights were measured.

Since anthracycline antibiotics can be adsorbed onto the walls of containers[17], glassware containing these solutions were silanized before use with dichloromethylsilane in toluene (3%) and subsequent rinsing with methanol.

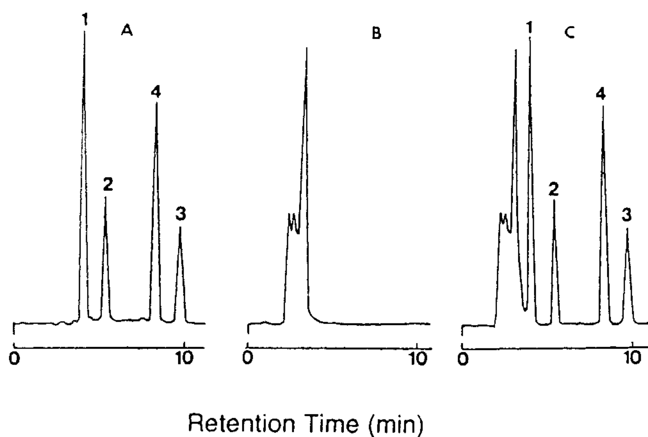


**Figure 3**. Chromatographic profiles of (A) standard ADR (2), ADR-OL (1), ADR-ONE (4), DAU (3); (B) drug-free human plasma; (C) plasma spiked with 20 nmol/mL tested anthracyclines.

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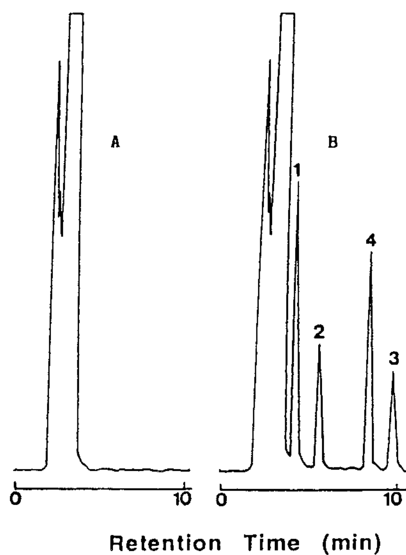


**Figure 4**. Chromatographic profiles of (A) drug-free human urine; (B) urine spiked with 20 nmol/mL of ADR (2), ADR-OL (1), ADR-ONE (4), DAU (3).

## RESULTS AND DISCUSSION

### Optimization of Experimental Variables

The effect of IP reagent in the mobile phase was studied by measuring the retention time of ADR, ADR-OL, ADR-ONE and DAU on ODS/TM column initially eluted with acetonitrile and 0.1 M monobasic phosphate (35 : 65, pH 3) and subsequently eluted with eluent of the same composition but with different concentrations of SHS and HFBA. Adriamycinol, the main metabolite of ADR could be separated from the parent compound under the first conditions (Figure 1 A). However, no adequate separation of ADR-OL in plasma could be obtained under these circumstances due to the interference caused by endogenous compounds (Figure 1 B). Similar findings were observed in the case of urine Figure 1 C).

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The effect of the nature of IP reagent and its concentration have shown that these parameters were very suitable for adjusting the degree of retention of ADR, ADR-OL, ADR-ONE and DAU (Figure 2). A strong selectivity change is observed between DAU and ADR-ONE and an improved resolution between ADR-OL and the endogenous components from plasma is obtained (Figure 3). Similar findings were observed in the case of urine (Figure 4).

Heptafluorobutyric acid 0.3% or SHS 0.5% together with 35% acetonitrile gave the best results for an optimum resolution and short time duration of the chromatographic separation. Due to the convenient properties of perfluorinated carboxylic acids, HFBA was recommended and used for all further experiments.

The influence of pH on the retention behavior of ADR, ADR-OL, ADR-ONE and DAU as well as endogenous compounds from plasma was investigated. It was noticed that, an increase in the hydrogen concentration ($pH < 5$) caused an increase in the retention time. At these pH values, the glycoside derivatives are ionized and IP formation is facilitated. In another word the amino groups would be protonated and thus would be amenable to IP formation with anionic IP reagents, which could be the cause of improving the base line separation. The optimum pH value of the mobile phase was 3 and was recommended in all further experiments.

The electrical potential at the stationary phase plays an important role in the retention of IP chromatography. This can be regulated not only by the IP reagent concentration, but also by the inorganic salt concentration in the mobile phase. Best resolution was obtained within the concentration range of 0.05-0.15 M. However, 0.1M monobasic phosphate solution was recommended because of the column and pump limitations for the high salt concentration.

### Table 1

### Statistical Data* of the Calibration Lines (Within-Day Values) for the Tested Anthracylclines from HPLC of Acetonitrile-Treated Spiked Plasma

| Compound | Slope | Intercept | r |
|---|---|---|---|
| ADR | 0.2689 | -0.0075 | 0.9996 |
| ADR-OL | 0.4654 | 0.0452 | 0.9997 |
| ADR-ONE | 0.3174 | -0.0290 | 0.9994 |
| DAU | 0.2040 | -0.0140 | 0.9992 |

*Ten concentrations ranging from 2.5 to 25 mmole/mL (n=6).

### Table 2

### Statistical Data* of the Calibration Lines (Within-Day Values) for the Tested Anthracylclines from HPLC of Directly Injected Spiked Urine

| Compound | Slope | Intercept | r |
|---|---|---|---|
| ADR | 0.2716 | -0.0031 | 0.9997 |
| ADR-OL | 0.4667 | 0.0076 | 0.9996 |
| ADR-ONE | 0.3141 | -0.0307 | 0.9997 |
| DAU | 0.2009 | -0.0065 | 0.9994 |

*Ten concentrations ranging from 2.5 to 25 mmole/mL (n=6).

### Table 3

### Recovery* for the Tested Anthracyclines from HPLC of Acetonitrile-Treated Spiked Plasma in the Absence of γ-CD

| Compound | $10^{-6}$ | | | $10^{-7}$ | | | $10^{-8}$ | | |
|---|---|---|---|---|---|---|---|---|---|
| | Rec. % | ±S.D | C.V.% | Rec. % | ±S.D. | C.V.% | Rec % | ±S.D. | C.V.% |
| ADR | 79.64 | 2.54 | 3.18 | 71.56 | 3.15 | 4.41 | 65.05 | 3.84 | 5.90 |
| ADR-OL | 84.35 | 2.48 | 2.94 | 81.62 | 2.82 | 3.46 | 78.82 | 3.25 | 4.12 |
| ADR-ONE | 88.27 | 2.25 | 2.55 | 85.30 | 2.61 | 3.06 | 81.62 | 3.03 | 3.71 |
| DAU | 80.70 | 2.30 | 2.85 | 75.13 | 2.98 | 3.96 | 68.05 | 3.67 | 5.40 |

*Values are % of spiked concentrations obtained from 500 μL plasma, based on peak heights (n=6).

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The role of organic modifier, which has the most pronounced effect on the retention behavior of test compounds, was studied. It was noticed that, decreasing the concentration of acetonitrile caused a sever increase in the retention time that accompanied by band broadening, while increasing the volume fraction of acetonitrile, caused a decrease in retention time of all tested compounds. Satisfactory results were obtained by using 35%(v/v) acetonitrile.

## Specificity

The specificity of the method was evaluated by analyzing drug-free human plasma and urine samples from several healthy volunteers. No interference from endogenous plasma constituents was observed at the retention times of ADR, ADR-OL, ADR-ONE and DAU (Figures 3,4).

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## Calibration Curves

Standard curves from HPLC of acetonitrile-treated spiked plasma and directly injected spiked urine samples were constructed by plotting the measured peak height versus concentration of drugs and metabolites. A linear relation between peak height and concentration existed over the range of concentration examined (2.5-25 nmol/mL) (Tables 1,2). The least detectable concentration was 0.025, 0.013, 0.019, and 0.028 nmol/mL of ADR, ADR-OL, ADR-ONE, and DAU, respectively.

## Recovery

Spiked drug-free human plasma samples for recovery studies were prepared by diluting the appropriate volume of standard solution of ADR, ADR-OL, ADR-ONE and DAU with blank plasma to make their concentrations at three definite levels, $10^{-6}$, $10^{-7}$, and $10^{-8}$ M. The spiked plasma was then carried through the deproteinization process. Comparison of peak heights from deproteinized sample with those from standard solutions was used to calculate the analytical recovery. It was noticed that, in the absence of K-CD, the percentage recovery by the present method using 50% (v/v) acetonitrile as deproteinizing agent was superior to that of 5% (v/v) trichloroacetic acid in view of its accuracy. Therefore, acetonitrile (50%) was recommended for protein precipitation throughout this work.

Enhancement of chemical stability of tested compounds during sampling procedure was achieved in the present work. The use of K-CD during sampling procedure provides a precise means of stabilization and leads to high recovery in quantitation of tested anthracyclines. The percentage recovery of spiked plasma

## Table 4

**Recovery* for the Tested Anthracyclines from HPLC of Acetonitrile-Treated Spiked Plasma in the Presence of γ-CD**

| Compound | $10^{-6}$ | | | $10^{-7}$ | | | $10^{-8}$ | | |
|---|---|---|---|---|---|---|---|---|---|
| | Rec. % | ±S.D | C.V.% | Rec. % | ±S.D. | C.V.% | Rec % | ±S.D. | C.V.% |
| ADR | 97.99 | 1.66 | 1.70 | 94.92 | 2.00 | 2.11 | 88.01 | 2.75 | 3.12 |
| ADR-OL | 98.56 | 1.48 | 1.50 | 96.74 | 1.94 | 2.00 | 93.01 | 2.14 | 2.30 |
| ADR-ONE | 99.85 | 1.25 | 1.25 | 98.63 | 1.95 | 1.97 | 96.56 | 2.35 | 2.43 |
| DAU | 99.87 | 1.82 | 1.82 | 95.64 | 2.03 | 2.13 | 88.30 | 2.71 | 3.07 |

*Values are % of spiked concentrations obtained from 500 μL plasma, based on peak heights (n=6).

## Table 5

**Recovery* for the Tested Anthracyclines from HPLC of Directly Injected Spiked Urine**

| Compound | $10^{-6}$ | | | $10^{-7}$ | | | $10^{-8}$ | | |
|---|---|---|---|---|---|---|---|---|---|
| | Rec. % | ±S.D | C.V.% | Rec. % | ±S.D. | C.V.% | Rec % | ±S.D. | C.V.% |
| ADR | 99.75 | 1.65 | 1.65 | 99.20 | 1.77 | 1.78 | 98.95 | 1.57 | 1.59 |
| ADR-OL | 100.40 | 1.51 | 1.50 | 99.71 | 1.53 | 1.53 | 99.37 | 1.81 | 1.82 |
| ADR-ONE | 100.04 | 1.33 | 1.33 | 99.89 | 1.46 | 1.46 | 99.61 | 1.47 | 1.47 |
| DAU | 99.35 | 1.43 | 1.44 | 99.33 | 1.51 | 1.52 | 99.07 | 1.66 | 1.67 |

*Values are % of spiked concentrations obtained from 500 μL plasma, based on peak heights (n=6).

following the described procedure in the absence of K-CD was low, especially in low concentrated samples (Table 3). Upon using 0.01 M K-CD, the stability of ADR, ADR-OL, ADR-ONE, and DNR was enhanced and subsequently the percentage recovery increased in all ranges of concentrations (Table 4). Also, it was observed that, the percentage recovery was affected by the initial drug concentration employed in the assay. Low percentage recovery was obtained from low concentrated sample ($10^{-8}$ M) and this was in agreement with the stability studies of ADR and other related compounds[18]

Since urine could be injected directly into ODS/TM column without sample preparation, the percentage recovery of spiked urine was almost quantitative (Table 5).

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## CONCLUSION

The presented analytical procedure allows an excellent resolution of ADR and its main metabolites ADR-OL and ADR-ONE as well as DAU and overcomes the interferences of the endogenous compounds from biological fluids. The sensitivity of both drugs and metabolites is adequate for determining their plasma or urine concentration after therapeutic doses. Therefore, it can be applied in clinical laboratories for measuring therapeutic drug levels and in research laboratories for investigation of pharmacokinetics of ADR and DAU.

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