Journal of Chromatography, 307 (1984) 323—333
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 2038

CONTINUOUS EXTRACTION OF URINARY ANTHRACYCLINE ANTITUMOR ANTIBIOTICS WITH THE HORIZONTAL FLOW-THROUGH COIL PLANET CENTRIFUGE*

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(First received June 28th, 1983; revised manuscript received December 15th, 1983)

SUMMARY

Extraction of doxorubicin (adriamycin) and daunorubicin and their metabolites from human urine was attempted utilizing the horizontal flow-through coil planet centrifuge. Partition coefficients of the drugs for various combinations of non-aqueous phases and aqueous salt solutions were determined. Optimal coefficients for adriamycin and daunorubicin were achieved with *n*-butanol—0.3 *M* disodium hydrogen phosphate. Extraction efficiencies of the drugs from human urine comparable to those obtained by standard resin column techniques could be realized by employing the *n*-butanol—urine (containing 0.3 *M* disodium hydrogen phosphate) system in the coil planet centrifuge, at flow-rates of 500—600 ml/h, and at 650 rpm revolutional speed. Small quantities of drugs and metabolites could be continuously concentrated into small volumes of the *n*-butanol phase from large volumes of salted urine. The versatility of the technique was demonstrated by its application to extraction of aclacinomycin A, a novel anthracycline antitumor agent, and its metabolites from human urine.

*Presented, in part, at the 1981 Pittsburgh Conference on Analytical Chemistry and Applied Spectroscopy, Atlantic City, NJ, U.S.A.

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0378-4347/84/$03.00

INTRODUCTION

Basic and clinical pharmacological studies on the metabolism, pharmacokinetics, and mechanisms of action of anthracycline antitumor antibiotics require extraction, concentration, and purification of parent drugs and their metabolites from aqueous media [1—6]. The drugs and metabolites of interest are often present in low concentrations in biological fluids, such as urine. Several techniques for extracting and concentrating anthracyclines from urine have been described [3—7]. Liquid—liquid partition methods offer advantages in ready availability and low cost of reagents, and in usually yielding an organic phase containing the extracted materials. However, conventional liquid partition techniques, such as batch methods [8] or the Craig counter current distribution method, require fairly constant operator attention and large volumes of solvent, which necessitate significant additional processing and concentrating of the extract before the materials of interest are obtained in a form suitable for further analyses. A method which minimizes these disadvantages is desirable.

A new countercurrent chromatographic system, utilizing a compact horizontal planet centrifuge, has been introduced [9]. Flow-through design afforded continuous extraction, permitting processing of large volumes of the mobile phase, and organic extracts of small volume were obtained. The capability of the apparatus was demonstrated by the extraction of dinitrophenylated amino acids into ethyl acetate from an aqueous medium containing sodium dihydrogen phosphate [10]. The method allowed recovery of the amino acids, without contaminants, in a short period of time and at high efficiency.

We applied the horizontal flow-through coil planet centrifuge to countercurrent extractions of anthracyclines and their metabolites from human urine.

MATERIALS AND METHODS

Reagents

Doxorubicin (adriamycin, ADR) and daunorubicin (DNR) (Fig. 1) were obtained through the Division of Cancer Treatment, National Cancer Institute, and used without further purification. Anthracycline aglycones were prepared by acid hydrolysis of ADR and DNR [1,6]. Other reagents were analytical grade, and used as received. Urine samples were collected in dark bottles, with 10 ml toluene per 3 l urine as preservative, and stored at 4°C.

Partition coefficients

Partition coefficients, defined as the ratio of the concentration of drug in the non-aqueous phase to that in the aqueous phase, were determined for ADR and DNR in biphasic solvent systems. Chloroform—isopropanol (3:1) mixture, ethyl acetate, and *n*-butanol were individually tested against 1.0 *M* solutions of various salts. Equal volumes of organic and aqueous phases were placed in a test tube, 100 nmol of drug were added, and the tube was vigorously mixed for 30 sec. Quantities of drug in each phase were measured by fluorometry (SPF-125, American Instrument, Silver Spring, MD, U.S.A.; excitation, 470 nm; emission, 585 nm).

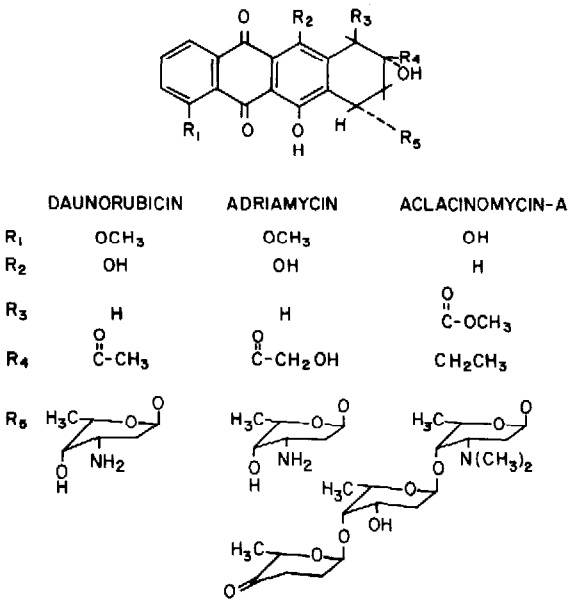

Fig. 1. Structures of adriamycin, daunorubicin, and aclacinomycin A.

Countercurrent chromatography

The design and principles of action of the horizontal flow-through coil planet centrifuge have been described in detail [9—12]. The chromatography column was prepared by winding PTFE tubing, 10 m × 2.6 mm I.D., around a holder 15 cm in diameter (column holder 1, Fig. 2); 18 helical turns were required. An appropriate counterweight was fixed to column holder II (Fig. 2). The capacity of the column was approximately 60 ml. The column was filled with *n*-butanol (stationary phase), and was subjected to planetary motion about two horizontal axes. The revolutional speed was 650 rpm. Disodium hydrogen phosphate (Na_2HPO_4) solution, 0.3 *M*, saturated with *n*-butanol, was pumped through the moving column to establish equilibrium conditions at 650 rpm. The stationary (butanol) phase volume was 32 ml. Prepared urine samples, 1—2 l in volume, were then pumped through the column at flow-rates of 500—700 ml/h, employing a multiple metering pump (Model 1508, Harvard Apparatus, Dover, MA, U.S.A.).

Urine was collected from patients or normal volunteers for 24 h during control periods, and patients for the first 24—48 h following anthracycline chemotherapy. Sufficient Na_2HPO_4 to yield a concentration of 0.3 *M* was added to several liters of urine. Salted urines were filtered (Whatman paper No. 802, Whatman, Clifton, NJ, U.S.A.) to remove precipitated material, and then saturated with *n*-butanol to prevent leaching of the columns's stationary phase. In some cases, filtration of the urine after butanol saturation was necessary. To test the efficiency of the countercurrent centrifuge extraction, known quantities of ADR and DNR were added to control urines. These spiked samples were processed in an identical fashion to post-therapy urines.

After each sample was chromatographed, the column was cleaned by elution with 100 ml of aqueous, *n*-butanol-saturated 0.3 *M* Na_2HPO_4. The *n*-butanol

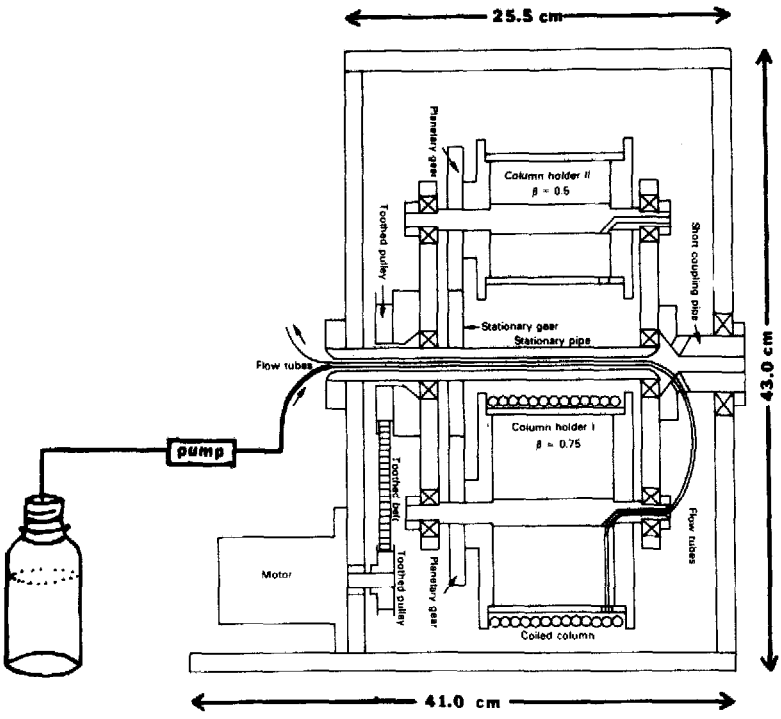

Fig. 2. Cross-section of the coil planet centrifuge. PTFE tubing is wound around column holder I (lower), and the counterweight is applied to column holder II (upper). A variable-speed pump delivers mobile phase from a large reservoir to the rotating coiled column while column effluent exits from the central stationary pipe as shown. The system eliminates the need for rotating seals. The dimensions of the apparatus, which is 45 cm deep, are indicated.

phase was drained from the column; several milliliters of *n*-butanol were passed through the column to recover any remaining sample. The *n*-butanol extracts were combined and evaporated to dryness by flash evaporation. The deep-red residue remaining after drying was redissolved in methanol.

Aliquots of pre-chromatography urine, column eluate (mobile phase), and the final extract from the column in methanol were analyzed for anthracycline content by fluorescence. A small volume of the aliquot was added to 2 ml of 1.8 *M* hydrochloric acid—ethanol (1:3), and concentration determined against known ADR standards in an Aminco-Bowman spectrophotofluorometer (American Instrument).

Solid—liquid chromatography

Urine samples identical to those subjected to countercurrent chromatography, but not prepared with Na_2HPO_4 or *n*-butanol, were studied by resin column chromatography. Glass columns, 250 × 11 mm, were packed with 150 mm of Amberlite XAD-2 (Rohm and Haas, Philadelphia, PA, U.S.A.). The chromatographic technique, a modification of the method of Fujimoto and co-workers [13,14], and preparation of the urine have been described [3,4]. Volumes (1 l) of patients' or spiked control urines containing ADR or DNR and their metabolites were chromatographed. Pre-chromatography urine,

column eluate, and final recovered samples were analyzed for anthracycline content as described above.

Thin-layer chromatography

Identification of parent anthracyclines and their metabolites in the sample fractions was accomplished with thin-layer chromatography (TLC). The methods have been previously detailed [3,4].

High-performance liquid chromatography

High-performance liquid chromatography (HPLC) was also employed to identify parent drugs and metabolites in the column fractions. The method has been detailed [7]. The apparatus included a Waters instrument [Model 6000A solvent delivery system, Model 660 solvent programmer, Model 440 detector, and phenyl-μBondapak column (10-μm particle size), 300 × 3.9 mm, Waters Assoc., Milford, MA, U.S.A.] with fluorescence detection (Aminco Fluoromonitor, American Instrument. Gradient elution with tetrahydrofuran in 0.45 *M* ammonium formate buffer was undertaken at a flow-rate of 1.5 ml/min.

RESULTS

Various salts were studied for their effects on partitioning of adriamycin and daunorubicin between aqueous and organic phases (Tables I and II). Three organic solvents were selected for study based on extensive previous experiences with anthracycline extractions: *n*-butanol [1,2,5,6], chloroform—isopropanol (3:1) [7,11,15], and ethyl acetate [2,6]. In nearly all cases, partitioning of

TABLE I

PARTITION COEFFICIENTS FOR ADRIAMYCIN

Adriamycin (100 nmol) was added to each 4.0-ml phase system. The concentration of aqueous salt solution was 1.0 *M* in all cases. The systems were vigorously mixed and allowed to equilibrate. Concentration of drug in each phase was measured by fluorometry, and partition coefficients were calculated as the ratio of the concentration in the non-aqueous phase to that in the aqueous phase. Insoluble salt crystals were noted in mixing 1.0 *M* Na_2HPO_4 with all non-aqueous phases, and no coefficient could be determined for the chloroform—isopropanol—Na_2HPO_4 system.

Salt	*n*-Butanol—water (1:1)	Chloroform—isopropanol—water (3:1:2)	Ethyl acetate—water (1:1)
None	2.1	2.3	0.1
$(NH_4)_2SO_4$	2.2	0.4	0.1
NaH_2PO_4	4.3	0.3	0.4
NH_4Cl	7.6	1.1	0.1
KCl	12.3	2.4	0.8
LiCl	10.0	1.8	0.7
CH_3COONH_4	10.1	4.1	0.4
NaCl	11.7	1.8	0.9
Na_2SO_4	10.8	7.7	0.1
Na_2HPO_4	>40.0	–	>1.2

TABLE II

PARTITION COEFFICIENTS FOR DAUNORUBICIN

Experimental conditions were as described in Table I. Daunorubicin (100 nmol) was added to each system. Insoluble crystals of Na_2HPO_4 were encountered with all non-aqueous phases, and no coefficient could be determined for the chloroform—isopropanol—Na_2HPO_4 system.

Salt	n-Butanol—water (1:1)	Chloroform—isopropanol—water (3:1:2)	Ethyl acetate—water (1:1)
None	2.7	3.2	0.1
$(NH_4)_2SO_4$	5.9	1.6	0.1
NaH_2PO_4	12.4	0.6	0.2
NH_4Cl	16.8	2.3	0.3
KCl	27.3	4.7	0.4
LiCl	20.9	4.4	0.3
CH_3COONH_4	21.6	12.1	1.3
NaCl	24.4	4.6	0.3
Na_2SO_4	36.3	12.5	0.6
Na_2HPO_4	>85.5	–	>6.9

anthracyclines into *n*-butanol was greater than into either of the other two systems. Partition coefficients were higher for DNR compared to ADR in nearly every biphasic system tested, because ADR is more polar than DNR (Fig. 1). The highest coefficients were obtained for the *n*-butanol—Na_2HPO_4 system at the 1.0 *M* Na_2HPO_4 concentration. However, precipitation of salt crystals was noted. Coefficients for a range of Na_2HPO_4 concentrations were measured

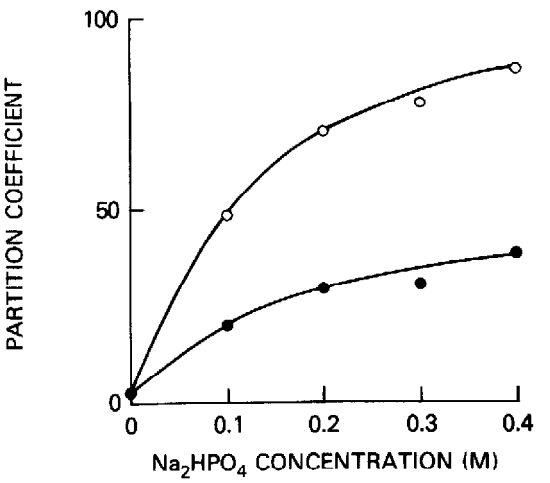

Fig. 3. Effect of Na_2HPO_4 on partition coefficients of adriamycin (•) and daunorubicin (○). Partition coefficients (ordinate) were measured in the *n*-butanol—aqueous Na_2HPO_4 system. Aqueous Na_2HPO_4 concentrations varied from 0—0.4 *M* (abscissa). A 100-nmol amount of drug was added to a test tube containing equal volumes of each phase, and the tube was vigorously mixed. The concentration of drug in each phase was measured by fluorometry, and the partition coefficients for adriamycin and daunorubicin were determined as ratios of concentrations in the non-aqueous and aqueous phases.

(Fig. 3), and a plateau was observed for concentrations greater than 0.4 *M*. No crystal formation occurred at Na_2HPO_4 concentrations less than 0.4 *M*. Further studies utilized the *n*-butanol—0.3 *M* Na_2HPO_4 system. At this Na_2HPO_4 concentration, partition coefficients for ADR and DNR were 31 and 78, respectively.

The capabilities of the technique were determined for extraction of daunorubicin and metabolites from a patient's urine (Fig. 4). A 24-h urine specimen (1.5 l) was collected from a patient who received DNR. Prior to countercurrent extraction, DNR and daunorubicinol (13-OH DNR) were the only identifiable drug species by reversed-phase HPLC; considerable interfering, non-anthracycline, polar materials were noted in the early column effluent (Fig. 4A). After preparation of the urine sample, as described in Materials and methods, the entire 1.5 l was chromatographed through the coil planet centrifuge. Only occasional operator attention was required to monitor pump and centrifuge conditions during the 3-h run. The retained *n*-butanol phase harvested from the column and two small *n*-butanol column washings were combined and evaporated to dryness. The residue was dissolved in methanol and subjected to

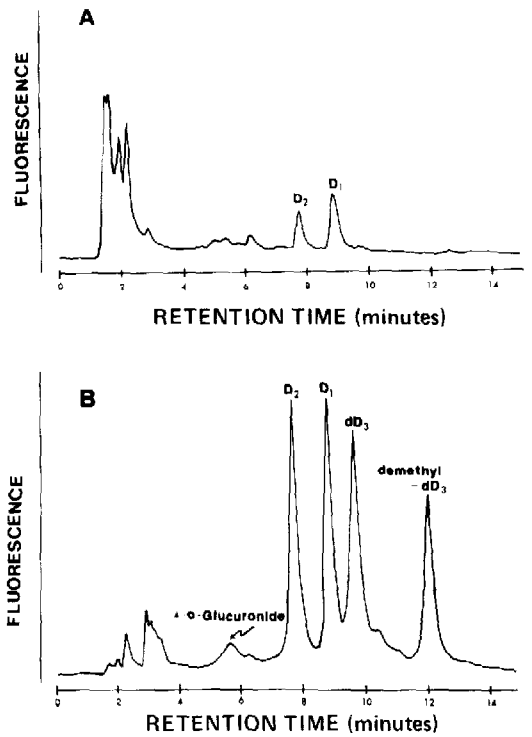

Fig. 4. Daunorubicin extraction from urine. (A) An aliquot of a 24 h urine sample from a patient treated with daunorubicin (DNR) was analyzed by reversed-phase HPLC. The early (2 min) column eluate contained considerable interfering materials. Only two anthracycline species, DNR (D_1) and 13-OH-DNR (D_2), were identifiable. (B) The same 24-h sample has been prepared and extracted by the coil planet centrifuge. The early interfering materials were largely excluded from the extract. Sharp, enriched peaks for D_1 and D_2 were evident, and three previously undetectable metabolites of DNR-1 conjugated species and two aglycones, dD_3 and demethyl-dD_3, were made apparent. HPLC conditions were given in Materials and methods; anthracycline detection was by fluorescence monitoring.

HPLC (Fig. 4B). In addition to DNR and 13-OH-DNR, which were greatly enriched in the extract, three metabolites not previously visible were demonstrable. The polar interfering materials had been largely eliminated from the extract. Similar enrichment could be shown for ADR and its metabolites (Fig. 5), although relatively greater amounts of interfering materials were recovered with the more polar ADR.

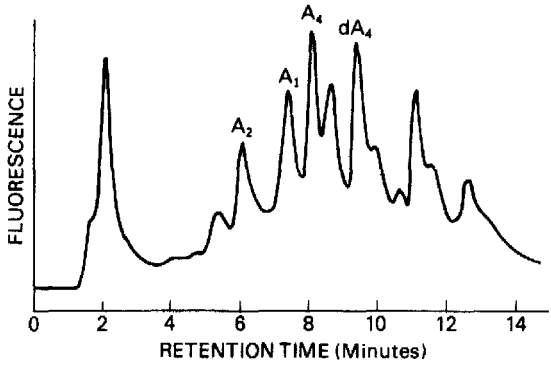

Fig. 5. Adriamycin extraction from urine. Urine from a patient receiving adriamycin was prepared, extracted, and chromatographed as in Fig. 4B. Adriamycin (A_1) and three metabolites (A_2, A_4 and dA_4) were enriched by coil planet centrifuge extraction. Increased amounts of polar interfering materials relative to the amounts of anthracycline were noted with adriamycin.

Aclacinomycin A (ACM), a novel anthracycline now undergoing clinical trials, [16,17], differs structurally from ADR and DNR both in the quinone system and the attached sugar moieties (Fig. 1). Urine was obtained from a patient who received ACM, and subjected to countercurrent extraction exactly as described for ADR and DNR. The *n*-butanol extract contained ACM and eight metabolites. The capacity factor (k') values calculated from reversed-phase HPLC values for these species are indicated in Table III [18].

Our standard technique for recovering anthracyclines from urine involves adsorption chromatography of pH-adjusted, filtered urine samples onto Amberlite XAD-2 resin [3,4]. We compared the XAD-2 method to the coil planet centrifuge for efficiency in extracting ADR and DNR from spiked control urines and authentic patients' specimens (Table IV). The XAD-2 technique yielded more efficient extraction of ADR than did the coil planet centrifuge, from spiked samples, but the methods were equivalent in their recoveries of the less polar DNR. The reverse was seen in the case of treated patients' urines, with about 50% improvement in recovery of ADR and metabolites using the coil planet centrifuge. This difference may have been due to altered partitioning of drug metabolites between the two phases, compared to parent drug. In particular, aglycone metabolites would be expected to distribute well into the *n*-butanol phase. Both TLC and reversed-phase HPLC confirmed that the coil planet centrifuge allowed recovery of relatively greater quantities of aglycones from urine than the XAD-2 column for ADR (60% aglycones with coil planet centrifuge versus 12% with XAD-2 column) and DNR (48% aglycones versus 6%).

TABLE III

CAPACITY FACTOR (k') VALUES FOR ACLACINOMYCIN A AND METABOLITES

Extracts of urine from ACM-treated patients were prepared by countercurrent chromatography with the coil planet centrifuge. The extracts were analyzed by reversed-phase HPLC as described in Materials and methods and k' values were calculated from the observed retention times. Aklavinone is the aglycone of ACM, and C_1, E_1, F_1, M_1, N_1, S_1, and aklavin are metabolites [18].

Compound	k'
F_1	8.9
E_1	9.8
C_1	8.2
Aklavinone	7.1
ACM	6.7
M_1	5.5
N_1	5.3
S_1	4.4
Aklavin	4.9

TABLE IV

COMPARISON OF ANTHRACYCLINE RECOVERIES FROM URINE

A. Adriamycin (ADR) or daunorubicin (DNR) was added to control urines which were then prepared and chromatographed as described in Materials and methods. The percentages of the added drug fluorescence which were recovered from the coil planet centrifuge or Amberlite XAD-2 column are displayed.
B. Urines from patients treated with ADR or DNR were chromatographed as in A. Recoveries, expressed as percentages of the initial anthracycline content, are indicated.

		Percentage recovery	
		Coil planet centrifuge	XAD-2 column
A.	ADR	27	65
	DNR	70	72
B.	ADR	63	44
	DNR	33	35

DISCUSSION

The horizontal flow-through coil planet centrifuge has been proven to be valuable for analytical chromatographic applications [9—11]. We have demonstrated in this paper that efficient recovery of anthracyclines from large volumes of urine by countercurrent extraction with the apparatus is feasible.

n-Butanol was chosen for the non-aqueous (stationary) phase on the basis of the favorable partition coefficients observed for ADR and DNR with nearly every aqueous salt phase. Technical considerations fortunately render *n*-butanol a desirable solvent because it contributes to low interfacial tension and lower density in biphasic systems [12]. These properties favor adequate mixing of

the phases and good retention in the column. Ethyl acetate is retained better in the rotating coil, but has higher interfacial tension and inferior partition coefficients compared to *n*-butanol.

Reversed-phase HPLC proved that meaningful purification of ADR or DNR from urine of treated patients can be achieved by the coil planet centrifuge. This has relevance both for clinical pharmacology and preparative applications. The identification and quantification of urinary anthracyclines is a necessary adjunct to pharmacokinetic studies on these drugs [2—6, 15]. Urine also remains the cheapest and most productive source of human anthracycline metabolites, which, when extracted and purified, are suitable as cochromatographic standards and as substrates for in vitro studies [1,3,4,7]. The versatility of the coil planet centrifuge is substantiated by its ready application to extraction of ACM and its several metabolites from human urine. Similar results, confirmed by HPLC and TLC, have been obtained with batch extractions of urine for ACM [19].

The coil planet centrifuge appears to extract polar materials less efficiently than Amberlite XAD-2, which is our current standard method (Table IV). Both HPLC and TLC demonstrate the presence of polar anthracyclines (e.g., ADR and the glucuronides of ADR and DNR) in XAD-2 or coil planet centrifuge extracts of urine. However, when the centrifuge eluate (mobile phase) is examined, relatively more polar species than aglycones of ADR or DNR are noted. We speculate that the stationary *n*-butanol phase in the coil planet centrifuge has a finite capacity to retain polar anthracyclines, which is less than its capacity for aglycones. Conversely, irreversible binding of aglycones to the Amberlite XAD-2 resin probably occurs, contributing to the reduced recovery of ADR or DNR aglycones observed in extracts from that technique. Both methods suffer from the need for filtration of urine prior to its application to the XAD-2 column or rotating centrifuge, resulting in increased processing time and cost. Anthracyclines bind avidly to the filter paper, resulting in an obligate lowering of recovery. This disadvantage can be partially avoided by employing glass fiber filters, but at additional expense.

The compactness, facility of operation, and observed extraction efficiencies of the coil planet centrifuge recommend that studies of its ability to recover other anthracyclines and drugs of other classes from urine be undertaken. Suitable alterations of the components of the biphasic system and inclusion of newer technologies, such as ion-pair solvent extractions [20], may permit better extractions of polar compounds. Efforts to increase the capacity of the stationary phase, such as manipulations of column bore or length, or speed of column revolution, are worthy of consideration.

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