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Anthracycline Assay by High-Pressure Liquid Chromatography

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Abstract □ A general method of analysis of anthracycline concentrations was developed. Drug is extracted from plasma with organic solvent and separated from metabolites by high-pressure liquid chromatography on an aminocyanosilica column. Detection and quantitation are by the endogenous fluorescence of compounds having an intact tetracyclic ring structure. Limits of sensitivity are 5, 1, and 5 ng/ml of plasma for doxorubicin, carubicin, and marcellomycin, respectively. The assay can be used for studying the aldo–keto reductase and reductive glycosidase reactions with anthracyclines as the substrates and for the evaluation of the clinical pharmacology or pharmacodynamics of various doxorubicin analogs.

Keyphrases □ Anthracyclines—doxorubicin, carubicin, daunorubicin, and marcellomycin, high-pressure liquid chromatographic analysis, dog plasma □ High-pressure liquid chromatography—analysis of doxorubicin, carubicin, daunorubicin, and marcellomycin in dog plasma □ Antineoplastic activity, potential—high-pressure liquid chromatographic analysis of anthracyclines in dog plasma, doxorubicin, carubicin, daunorubicin, and marcellomycin

Doxorubicin (adriamycin) is the best known of several hundred characterized anthracyclines. It has a broad spectrum of activity, so it is widely used in cancer therapy. However, because of significant acute and chronic toxicities, there is a constant search for active anthracyclines with less severe or less frequent adverse effects.

Several anthracycline analogs have reached the clinical stage of development. Daunorubicin (daunomycin, cerubidine), for example, was shown to be useful in the treatment of leukemia (1) and is now commercially available. Carubicin (carminomycin), an anthracycline that was developed and clinically tested in the Soviet Union (2), is being evaluated in the United States and Europe. Aclacinomycin A, a drug from Japan, is being evaluated in Europe (3) and tested clinically in the United States. Another anthracycline, marcellomycin, appears to be relatively nontoxic to white cells in animals (4) and is now undergoing toxicity studies in preparation for clinical testing.

A simple, rapid, inexpensive method of analysis of anthracycline concentrations in biological fluids is needed not only for comparative studies of pharmacokinetics and pharmacodynamics but also for the clinical monitoring of patients receiving doxorubicin analogs. A method for determining parent drug concentrations for various anthracyclines including doxorubicin, daunorubicin, carubicin, and marcellomycin has been developed. The method employs drug extraction with an organic solvent followed by high-pressure liquid chromatography (HPLC) with fluorescence detection. *In vitro* enzyme studies and *in vivo* pharmacokinetic investigations can be performed using this assay.

EXPERIMENTAL

Reagents and Drugs—Purified doxorubicin[1], daunorubicin[1], carubicin[2], marcellomycin[2], rudolphomycin[2], and aclacinomycin A[2] were used for assay development. Clinical grade doxorubicin[3] and carubicin[2] were used in animal studies. Adriamycinol and daunorubicinol were obtained by reduction of the parent compound with aldo–keto reductase according to the procedure of Felsted *et al.* (5). Carminomycinol was prepared by borohydride reduction according to a modified procedure of Povarov *et al.* (6).

Aglycones were prepared by acid hydrolysis at elevated temperature (7). Deoxyaglycones of doxorubicin and adriamycinol were produced by reaction of the parent compound with microsomal enzymes in the presence of reduced nicotinamide-adenine dinucleotide phosphate (NADPH) under anaerobic conditions (8). Solvents were reagent grade or better, and degassing was not necessary. Water was glass distilled.

Instruments—Volume measurements were made with automatic pipets[4]. The high-pressure liquid chromatograph included an injection port[5] and a single-piston pump[6] with electronic damping. The column (25 cm × 4.6 mm i.d.) was packed with 10-μm aminocyanosilica[7]. The detection unit consisted of a filter-type fluorometer[8] with an emission filter of bandwidth 560–570 nm and an excitation filter of bandwidth

[1] Adria Laboratories, Dublin, Ohio.
[2] Bristol Laboratories, Syracuse, N.Y.
[3] Adriamycin, Adria Laboratories, Dublin, Ohio.
[4] Digital Pipetman, Rainin Instrument Co., Brighton, Mass.
[5] Model 7210, Rheodyne, Berkeley, Calif., or model U6K, Waters Associates, Milford, Mass.
[6] Model 110, Altex, Rainin Instrument Co., Brighton, Mass.
[7] Partisil-10 PAC, Whatman, Clifton, N.J.
[8] Spectra-Glo, Gilson Medical Electronics, Middleton, Wis.

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380–480 nm equipped with a 15-μl flowcell. Chromatograms were recorded on a chart recorder with an electronic integrating channel[9].

Assay of Plasma Samples—Plasma, obtained from heparinized blood, was taken from storage at −10° or colder and warmed to room temperature. Samples then were vortexed rapidly or sonicated to disperse the cryoprecipitate. Aliquots (1.0 ml) were transferred to 15-ml tubes[10], and a small volume (20 μl) of the internal standard in aqueous solution was added. The internal standard consisted of an anthracycline with a convenient retention time that did not interfere with the peaks secondary to the test drug or its metabolites. Table I lists the internal standards used with the various test compounds.

To each plasma sample, two volumes of chloroform–isopropanol (1:1 v/v) solution were added. The mixture was vortexed for 20 sec, saturating quantities of ammonium sulfate were added, and the mixture was vortexed again. The tubes were centrifuged at 2650×*g* for 30 min, and the upper organic layer was removed and placed in clear, glass tubes. The organic solvent was evaporated under a nitrogen gas stream in a temperature-controlled water bath at 37°. The residue was analyzed immediately or stored at −40° until the next day. The residues were dissolved in 50 μl of the mobile phase just prior to injection into the chromatograph.

The column was conditioned before the first injection of the day by eluting with chloroform–methanol (1:1) at 1.5 ml/min for 30 min and then with the mobile phase of chloroform–methanol–acetic acid–water (80:20:2:3) at the operating flow rate of 3.5 ml/min for 30 min. This conditioning was reversed for the shutdown procedure, and a final wash of 100% methanol for 30 min at 1.5 ml/min was used. A constant flow rate of 3.5 ml/min was maintained during analysis at a column head pressure of 2700–4500 psi. When pressure greater than 4500 psi was encountered, the filter frits at the input end of the column were changed.

Standards encompassing the range of concentrations expected from plasma samples were run at the beginning of each day. Standards were prepared by spiking pooled dog plasma with the test drug and internal standard and carrying them through the assay. Peak heights of the test drug and internal standard were compared directly without correction for retention time or by comparison of the area under the peaks. Peaks were sharp so little difference between these methods was noted. Peak heights were normalized by dividing the peak height by the relative gain of the fluorescence detector. This procedure required changing the sensitivity setting when peaks started to go off scale.

***In Vitro* Metabolism Studies**—Enzyme kinetic studies of daunorubicin reductase (aldo–keto reductase) and daunorubicin reductive glycosidase (cytochrome P-450 reductase) were performed according to Bachur and associates (5, 8, 9), except that TLC was replaced by HPLC separation. The method involved incubation of the substrate with reduced nicotinamide-adenine dinucleotide phosphate in a suitable buffer with the enzyme. The product of the aldo–keto reductase reaction was adriamycinol when doxorubicin was the substrate. When the reductive glycosidase was incubated under anaerobic conditions, deoxyadriamycin aglycone was the product. The reaction was stopped by thorough mixing with *n*-butanol and saturating quantities of solid sodium chloride.

An aliquot (usually 20 μl) of the butanol layer, which extracted drug and metabolites, was injected directly into the chromatograph. The normalized peak height secondary to the fluorescence of the major metabolite was expressed as a fraction of the summation of all normalized peak heights. This fraction was multiplied by the initial amount of substrate to give the quantity of product formed. Since the tetracyclic ring structure of the anthracycline remains intact following either enzymatic conversion, it was assumed that the molar fluorescence of the substrate and products was the same and that no significant quantities of nonfluorescent products were formed.

RESULTS AND DISCUSSION

The retention times of various anthracyclines and other fluorescent compounds are given in Table II. Because of slight changes in retention times with column maturation, authentic samples of compounds were sometimes injected into the column to confirm that a particular peak was due to the drug or metabolites and not to a spurious peak secondary to an unknown fluorescent plasma constituent.

Standard curves for doxorubicin, carubicin, and marcellomycin were linear over the range tested when four or five concentration points were used. The standard curve for doxorubicin with daunorubicin (250 ng/ml) as the internal standard was linear to 1250 ng/ml with a correlation coefficient of 0.997 for the best-fit line. The doxorubicin curve can be based on the area under the peak or normalized peak heights with similar results. The doxorubicin–daunorubicin system was reproducible from day to day with an average standard curve slope of 2.54 and a coefficient of variation of 10% ($n = 4$).

The standard curve for carubicin with doxorubicin (400 ng/ml) as the internal standard was linear to 50 ng/ml with a correlation coefficient of 0.994 for five concentration points done in triplicate. The marcellomycin standard curve was prepared using doxorubicin (400 ng/ml) as the internal standard and was linear to 100 ng/ml with a correlation coefficient of 0.991 for four concentration points done in triplicate. Standard curves for carubicin and marcellomycin were as reproducible as those for doxorubicin. Usually standard curves were generated at the beginning of test runs using three to five samples. Limits of sensitivity were 5, 1, and 5 ng/ml of plasma for doxorubicin, carubicin, and marcellomycin, respectively.

Plasma disappearance curves of doxorubicin and carubicin are shown in Fig. 1. Each curve was generated by giving the drug to a dog by rapid intravenous injection and obtaining plasma samples at timed intervals. Although the doxorubicin dose was 12-fold that of carubicin, plasma concentrations of doxorubicin at later time points were about 25 times higher than carubicin levels. The slopes of the terminal phases of both drugs in the two dogs appeared similar with half-lives of ~20 hr.

The enzyme kinetics of daunorubicin reductase and reductive glycosidase are shown in Figs. 2 and 3, respectively. The reactions were linear with respect to time over 15 min when protein concentrations were <1.5 mg/ml for the reductase and <0.4 mg/ml for the glycosidase. A Lineweaver–Burk reciprocal plot for the daunorubicin reductase also is shown in Fig. 2. The value obtained for the K_m of this enzyme was approximately the same as was reported previously (5).

The use of HPLC with fluorescence detection for the isolation and separation of anthracyclines is an established technique (10–19). Many methods described in the literature involve multistep extraction procedures, lack sensitivity, use expensive instrumentation, or require gradient elution. The method described in this paper is actually the extraction method used by Benjamin *et al.* (20) and the HPLC methodology of Harris and Gudauskas (11). The instrumentation was chosen on the basis of simplicity, reproducibility, and cost. Since fluorescence detection is

Table I—Internal Standards Used with Test Compounds in the Anthracycline Assay

Test Compound	Internal Standard (Concentration)
Doxorubicin	Daunorubicin (250 ng/ml) or carubicin (50 ng/ml)
Daunorubicin	Doxorubicin (250 ng/ml)
Marcellomycin	Doxorubicin (400 ng/ml)
Aclacinomycin A	Doxorubicin (400 ng/ml)
Carubicin	Doxorubicin (400 ng/ml)

Table II—Retention Times for Various Anthracyclines and Other Fluorescent Compounds[a]

Compound	Retention Time, min
Solvent front	0.5
Adriamycin aglycone	0.5
Carminomycin aglycone	0.5
Daunorubicin aglycone	0.5
Pyrromycinone	0.5
Quinine sulfate	0.8
Acridine	1.0
Aclacinomycin A	1.1
Pyrromycin	1.7
Bleomycin	2.0
Marcellomycin	2.0
Daunorubicin	2.2
Rudolphomycin	2.2
Carubicin	2.3
Proflavin	2.4
Puromycin	2.5
Musettamycin	2.6
Carminomycinol	3.3
Doxorubicin	3.9
Adriamycinol	4.5
Quinidine sulfate	4.8

[a] The column was Whatman Partisil-10 PAC, the eluting solvent was chloroform–methanol–acetic acid–water (80:20:2:3), and the flow rate was 3.5 ml/min.

[9] Linear No. 252, Serco, Deerfield, Ill.
[10] Corex, Kimble Products.

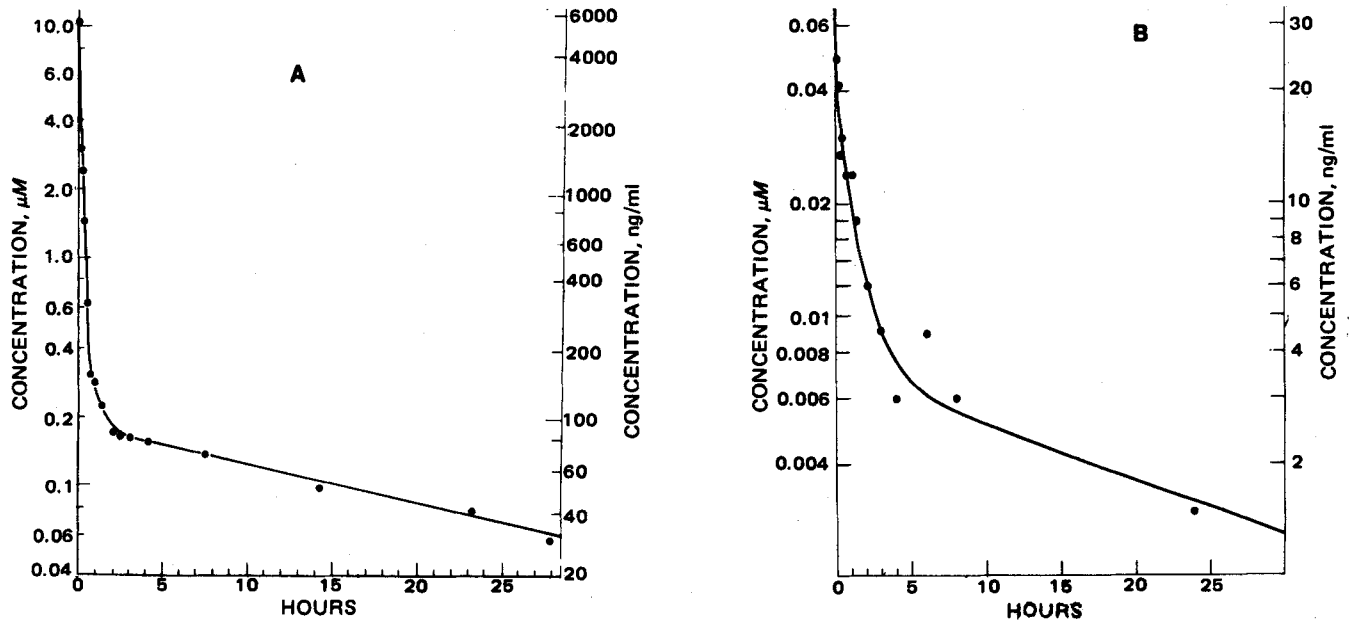

Figure 1—*Plasma disappearance curves for anthracyclines given to a dog as a bolus intravenous injection. Key: A, doxorubicin (90 mg/m²); and B, carubicin (7.5 mg/m²).*

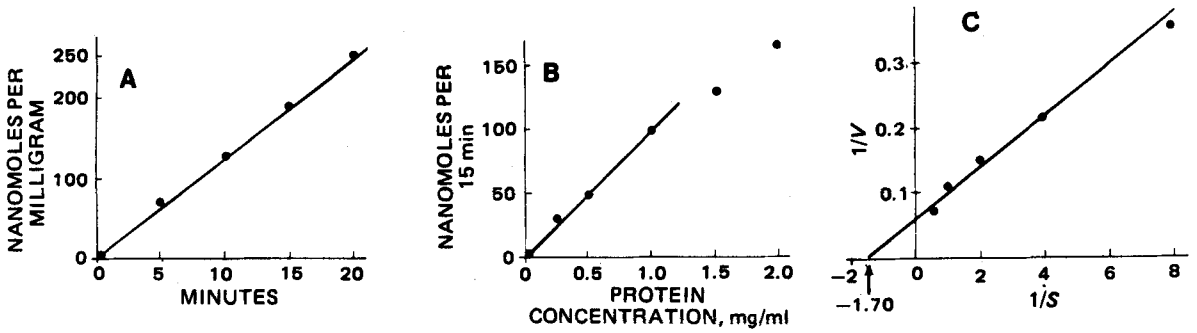

Figure 2—*Kinetics of the aldo–keto reductase enzyme with daunorubicin as the substrate. Final concentrations in 0.5 ml were 0.05 M phosphate buffer (pH 7.4), 1 × 10⁻³ M NADPH, 0–2 mg of protein/ml, and 1 × 10⁻³ M daunorubicin. Key: A, nanomoles of daunorubicinol per milligram of protein formed versus time; B, nanomoles of daunorubicinol formed per 15 min versus protein concentration; and C, Lineweaver–Burk reciprocal plot (K_m = 59 μM).*

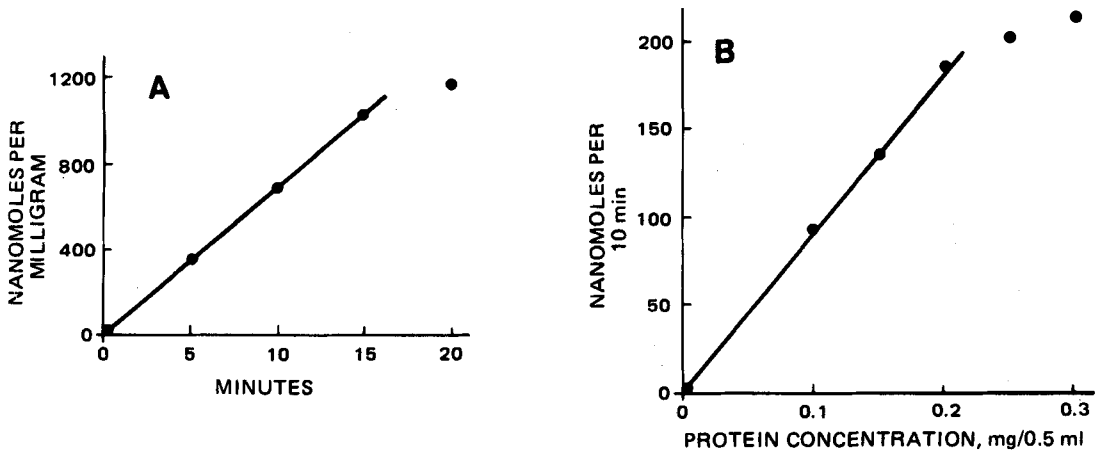

Figure 3—*Kinetics of reductive glycosidase with daunorubicin as the substrate. Final concentrations in 0.5 ml were 0.05 M phosphate buffer (pH 7.4), 1 × 10⁻³ M NADPH, 0–0.6 mg of protein/ml, and 1 × 10⁻³ M daunorubicin. Key: A, nanomoles of deoxydaunomycinone per milligram of protein formed versus time; and B, nanomoles of deoxydaunomycinone formed per 10 min versus protein concentration.*

not sensitive to pressure changes, the pulsation caused by the single-piston pump used in this system does not affect the chromatogram.

One disadvantage of this method is that the number of samples that can be processed is limited to about six or seven per hour since 6–10 min is required between injections. However, it takes about the same time to develop, scrape, and quantify materials on TLC plates, and TLC of doxorubicin may cause artifactual metabolite concentration values (21). Another disadvantage is that aglycones cannot be readily quantified from

plasma since aglycones of the drug and metabolites are not well resolved and elute near the solvent front, where there are high levels of nonspecific fluorescence due to plasma constituents.

Aglycone separation is not a problem in the *in vitro* assays since there are few interfering substances. Separation of aglycones from deoxyaglycones can be accomplished for most anthracyclines using the aminocyanosilica column if a less polar mobile phase is used. The parent compound, however, will have a longer retention time under these conditions.

Marcellomycin is an anthracycline with a three-sugar chain linked by a glycosidic bond to pyrromycione (22). Mussettamycin and pyrromycin are the di- and monosaccharides in this series, respectively. As can be seen from Table II, these four anthracyclines can be separated easily by this system.

The relative peak heights of test compounds compared to doxorubicin in this HPLC system depend on three major factors: (*a*) the molar fluorescence of the compounds at the excitation–emission bandwidths used in the fluorescence detector, (*b*) the extraction efficiency of the chloroform–isopropanol solvent, and (*c*) the retention time of the compound. Since retention times of the compounds are short, there is not much spreading of peak widths so the third factor does not influence peak heights substantially. However, the other two factors are important since the relative fluorescence of carubicin is ~2.5–3 times that of doxorubicin in this system. For example, 50 ng of carubicin/ml produces a peak height that is ~33% of that due to 400 ng of doxorubicin/ml. This result means that the fluorescence due to 50 ng of carubicin is equivalent to that due to 132 ng of doxorubicin/ml. Since the standard curve for carubicin is linear, it is not necessary to determine if this greater relative fluorescence is due to differences in molar fluorescence or to extraction efficiency. The relative fluorescence of doxorubicin, daunorubicin, and marcellomycin appears to be comparable when these drugs are extracted from pooled plasma.

The examples reported here demonstrate the usefulness and versatility of HPLC to anthracycline research. There is good evidence that doxorubicin toxicity can be predicted on the basis of elevated drug exposure or the area under the plasma concentration curve (23). If this observation holds for anthracycline analogs, then drug monitoring could warn against toxicity during early clinical trials. This HPLC system would then be useful in obtaining concentrations within a reasonable time with accuracy and reproducibility.

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ADDENDUM TO ANTHRACYCLINE ASSAY BY HIGH-PRESSURE LIQUID CHROMATOGRAPHY: MODIFICATION OF MOBILE PHASE FOR AMINOCYANOSILICA COLUMNS USED FOR ANTHRACYCLINE ASSAY

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High-pressure liquid chromatography (HPLC) is an efficient technique for the separation and quantification of anthracycline antibiotics. Various assay procedures have been described, including several that employ an aminocyanosilica column[3] (1–3). Users of this column should be aware that a modification in the manufacturing process for the packing material has changed the properties of this column with respect to anthracycline assay. Although the change improved the uniformity of the columns without significantly altering retention times of standard test compounds or the number of theoretical plates, the affinity of the packing for anthracyclines was decreased. No other problems secondary to the change have been reported to the manufacturer.

Table AI—Retention Times for Several Compounds [a]

Compound	Retention Time, min
Solvent front	1.3
Acridine	1.3
Aclacinomycin A	1.3
Carubicin	3.0
Quinidine sulfate	3.3
Daunorubicin	3.6
Puromycin	3.6
Quinine sulfate	3.8
Carminomycinol	4.0
Proflavin	4.0
Daunorubicinol	4.8
Doxorubicin	6.3

[a] The column was Whatman Partisil PXS 10/25 PAC (lot 100320), the mobile phase was hexane–ethylene chloride–methanol–acetic acid–water (40:40:10:4:1), and the flow rate was 3.0 ml/min.

Those columns[3] manufactured prior to May 24, 1979, separate doxorubicin (adriamycin), adriamycinol, adriamycin aglycones, daunorubicin, and daunorubicinol with a mobile phase of choloform–methanol–acetic acid–water (80:20:2:3). Columns manufactured after May 1979 (lots 100302 and higher) require a modification in the mobile phase to separate these compounds; hexane–ethylene chloride–methanol–acetic acid–water (40:40:10:4:1) at a flow rate of 3.0 ml/min can be used without loss of separation or sensitivity compared to the old system.

To document that the new system can be used for assay of doxorubicin and other anthracyclines, standard curves for doxorubicin and carubicin (carminomycin) were prepared in plasma. Daunorubicin (50 ng/ml) and

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[2] Whatman, Clifton, NJ 07014.
[3] Partisil-10 PAC, Whatman, Clifton, NJ 07014.

doxorubicin (150 ng/ml) were used as the internal standards, respectively. Samples were assayed according to the procedure of Averbuch *et al.* (3), except that new columns (lot 100302) were used with the hexane–ethylene chloride–methanol–acetic acid–water mobile phase system. The assay sensitivity (signal strength of >2.5 times baseline noise) was 5 and 1 ng/ml, respectively. In both cases, the response was linear to 200 ng/ml (highest concentration tested). The correlation coefficient for six points was >0.99 for both assays. Retention times for several anthracyclines and other fluorescent compounds were determined (Table AI).

The aminocyanosilica column is effective in separating highly polar compounds and is almost free of solvent memory. It is unfortunate that a change in silica gel manufacture caused a substantial change in operating characteristics with respect to the anthracycline assay. However, if the appropriate mobile phase is used, depending on lot number, then this column continues to be useful to anthracycline research.

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Effect of Surfactant on Tetracycline Absorption across Everted Rat Intestine

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Abstract □ Absorption of tetracycline hydrochloride (500 μg/ml) from oxygenated modified Krebs buffer in randomized everted rat jejunal segments was determined alone and in the presence of calcium, polysorbate 80, and calcium plus polysorbate 80. Surfactant increased absorption of tetracycline in the presence and absence of calcium, with 0.01% (w/v) polysorbate 80 increasing transfer to the greatest extent of the concentrations examined (0.005, 0.01, 0.05, 0.1, and 1%); tetracycline hydrochloride + 12.5 m*M* $CaCl_2$, 143 ± 45 μg/ml; tetracycline hydrochloride + polysorbate 80, 389 ± 18 μg/ml; tetracycline hydrochloride + 12.5 m*M* $CaCl_2$ + polysorbate 80, 255 ± 31 μg/ml. On the premise that the effective surfactant concentration is similar to the critical micelle concentration, an absorption mechanism based on micellar solubilization is postulated.

Keyphrases □ Tetracycline—absorption, effect of polysorbate 80, everted rat intestine □ Polysorbate 80—effect on tetracycline absorption across everted rat intestine □ Surfactants—effect of polysorbate 80 on tetracycline absorption across everted rat intestine

Tetracycline is a widely used antibiotic. Its serum level should not fall below the minimum inhibitory concentration (MIC), usually between 0.1 and 1.5 μg/ml, for most bacterial strains. Occasionally, this concentration is difficult to maintain due to decreased absorption caused by other drugs or divalent and trivalent cations present in the GI tract (1–3). The intestinal absorption of most tetracyclines is rapid but incomplete, and the absorption mechanism is poorly understood (4, 5).

Physicochemical interactions in the gut are not uncommon and, at least pharmacokinetically, seem to be the most important form of interaction affecting the absorption of tetracycline derivatives. Chelation of tetracycline with polyvalent cations, which has been well documented, is the most common reaction that decreases tetracycline absorption. In addition to forming direct tetracycline–metal complexes, tetracycline binds to both nucleic acids and proteins, with the binding mediated by divalent cations such as zinc, calcium, and magnesium.

Recently, the role of endogenous and exogenous surfactants in drug absorption experiments was explored (6, 7). In some instances, the addition of surfactants enhanced drug absorption (8).

Since decreased tetracycline absorption presents a clinical problem, often requiring alteration of food and medications, a method of optimizing tetracycline absorption to avoid the present restrictions would be valuable. Surfactants may enhance tetracycline absorption to an acceptable level, even in the presence of divalent ions; if so, they may prove to be a valuable additive to oral dosage forms.

This investigation determined the effects of surfactants on tetracycline absorption from the rat everted gut sac to obtain information on the enhancement of tetracycline transport and the tetracycline transport mechanism.

EXPERIMENTAL

Intestine Preparation—An everted intestinal sac technique (9, 10) was used. The solution on the inside of the sac is referred to as the serosal solution, and the solution in which it is incubated is called the mucosal solution.

Seven male white Holtzman rats, 175–200 g, were fasted overnight (20–24 hr), but water was not withheld prior to the experiment. The animals were anesthetized with ether and then killed by stunning and cervical fracture. The jejunum was removed and rinsed immediately with several portions of cold normal saline and everted on a thin glass rod. After eversion, the jejunum was washed in cold normal saline and cut into seven segments of 5-cm length. Segments of the rat intestine were selected randomly for the different treatment groups.

Sacs of everted intestine were prepared by tying one end tightly and the other end loosely with fine thread. Then 0.5 ml of buffer was introduced inside the sac with a 1-ml syringe fitted with a blunt needle. The loose ligature was tightened, and the buffer-filled sacs then were transferred immediately to 25-ml erlenmeyer flasks containing 15 ml of buffer mixed with tetracycline hydrochloride[1], 0.5 g/liter with or without the additives. Control sacs were placed in buffer alone. The other five sacs were incubated in buffer to which 0.005, 0.01, 0.05, 0.1, or 1% (w/v) pol-

[1] Lot 6 X090-71000, Pfizer Laboratories, New York, N.Y.

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