

Antiproliferative Activity of Doxorubicin and Aminoanthraquinone Derivatives on Chinese Hamster Ovary Cells

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Abstract □ A study of the antiproliferative activity of doxorubicin and several substituted aminoanthraquinone derivatives on Chinese hamster ovary cells was conducted. Doxorubicin and a derivative each inhibited cell proliferation at low concentrations, the latter being more potent than doxorubicin. A structure-activity relationship of these compounds is discussed in connection with an earlier postulated N-O-O triangulation hypothesis.

Keyphrases □ Aminoanthraquinone derivatives—synthesis and evaluation for antiproliferative activity, Chinese hamster ovary cells □ Doxorubicin—substituted aminoanthraquinone derivatives synthesized and evaluated for antiproliferative activity, Chinese hamster ovary cells □ Antiproliferative activity—aminoanthraquinone derivatives synthesized and evaluated for activity in Chinese hamster ovary cells □ Structure-activity relationships—substituted aminoanthraquinone derivatives synthesized and evaluated for antiproliferative activity

Based on a working hypothesis involving the recognition of a common N-O-O triangular pharmacophore among several antineoplastic agents (1, 2), 1,4-dihydroxy-5,8-bis[2-(2-hydroxyethyl)amino]ethylamino-9,10-anthracenedione (I) was synthesized and found to possess outstanding antineoplastic activity in several experimental tumor systems (3, 4). Recently, a number of structural modification (5, 6), structure-activity (5, 7), pharmacological (7, 8), mechanistic (9, 10), and clinical trial (11) experiments were conducted. Since several analogs of I exhibited antineoplastic activity *in vivo* but do not contain the entire N-O-O triangular pattern, an *in vitro* study of their antiproliferative activity on Chinese hamster ovary cells was undertaken to obtain additional structure-activity relationship information.

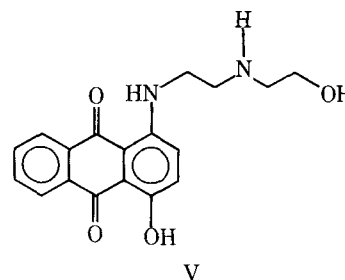
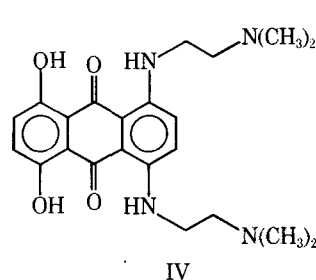
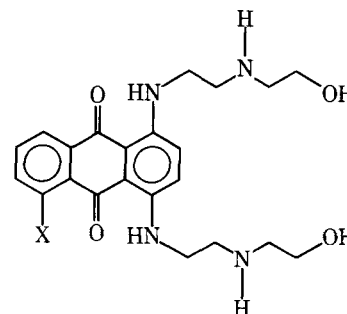
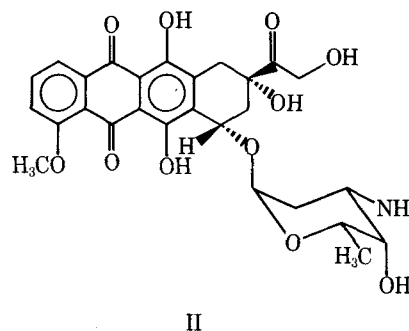
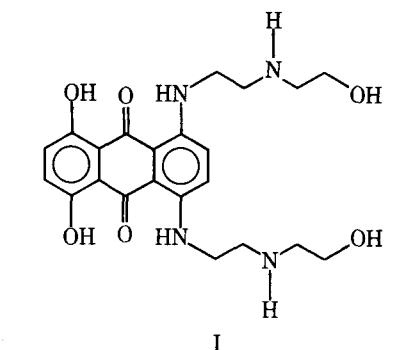
EXPERIMENTAL

Chemical Agents—Seven compounds, including the antibiotic doxorubicin¹ (II), were evaluated. Preparation of I, IIIa, IV, and V was reported previously (3, 5).

Preparation of 1-Amino-5,8-bis[2-(2-hydroxyethyl)amino]ethylamino-9,10-anthracenedione (IIIb)—To a mixture of 8 g (0.031 mole) of 5-amino-2,3-dihydro-1,4-dihydroxy-9,10-anthracenedione (prepared by the reduction of 5-nitro-1,4-dihydroxy-9,10-anthracenedione in zinc and acetic acid) in 55 ml of 2-propanol at 50° was added 30 g (0.29 mole) of 2-[(2-aminoethyl)amino]ethanol with mechanical stirring under nitrogen. The resulting reaction mixture was stirred at 50° for 4.5 hr, during which time a precipitate formed. The mixture was cooled to room temperature and filtered.

The solid was washed with 2-propanol and ether and dried to give 8.3 g (60% yield) of the dihydro analog of the target compound, 1-amino-5,8-bis[2-(2-hydroxyethyl)amino]ethylamino-9,10-dihydroxyanthracene, as gold-colored plates. Recrystallization from 2-propanol yielded an analytical sample, mp 135–145° dec.; UV: λ_{\max} (0.1 N HCl) 488 (ϵ 9800), 460 (15,300), 435 (11,700), and 252 (25,200) nm; λ_{sh} (0.1 N HCl) 280 (ϵ 17,000) nm.

Anal.—Calc. for $\text{C}_{22}\text{H}_{31}\text{N}_5\text{O}_4$: C, 61.52; H, 7.28; N, 16.31. Found: C,



61.35; H, 7.11; N, 16.12.

The mother liquor from the initial filtration was allowed to stir uncovered for 10 days at room temperature. The solution gradually turned dark blue and deposited a blue gummy solid. Filtration of this material

¹ Supplied by Dr. Harry B. Wood, Jr., National Cancer Institute.

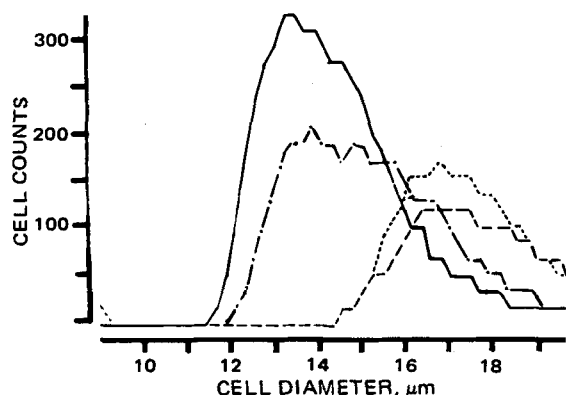


Figure 1—Cell counts and cell-sizing data for I. The data were stored and processed in a microprocessor. Every point of each line represents the average value of I on triplicate cultures. Key: —, control; --, 1000 nM; ····, 200 nM; and - · - ·, 10 nM.

and recrystallization from a mixture of methanol and 2-propanol gave 0.5 g (4% yield) of IIIb as a dark blue-black amorphous powder, mp 131–141° (dampening at 110°); UV: λ_{max} (0.1 N HCl) 634 (ϵ 14,400), 590 (13,500), and 252 (27,500) nm; λ_{sh} (0.1 N HCl) 550 (ϵ 7500) and 272 (14,500) nm.

Anal.—Calc. for $\text{C}_{22}\text{H}_{29}\text{N}_5\text{O}_4$: C, 61.81; H, 6.84; N, 16.38. Found: C, 61.53; H, 6.81; N, 16.04.

Compound IIIb could be obtained also from the dihydroanthraquinone product as follows. To 100 ml of refluxing nitrobenzene with magnetic stirring was added 5.0 g of the dihydroanthraquinone. After 20 min, the reaction mixture was allowed to cool, and 200 ml of ether was added. Filtration and drying of the solid product gave 3.4 g of IIIb.

Preparation of 1-Hydroxy-5,8-bis[2-(2-hydroxyethyl) amino]ethylamino-9,10-anthracenedione (IIIc)—To 30 g (0.29 mole) of 2-[(2-aminoethyl)amino]ethanol at room temperature under nitrogen was added 8.5 g (0.033 mole) of 1,4,5-trihydroxy-2,3-dihydroanthraquinone in one portion with stirring. A slight exothermic reaction developed. The reaction mixture was stirred at room temperature for 3 hr and then was warmed at 50° for 1 hr. To this mixture was added 250 ml of 2-propanol, and the dark solution again was warmed to 50°. Air was bubbled into the reaction mixture for 4 hr at that temperature, and then it was cooled to 30° and filtered.

The solid was washed with 2-propanol followed by ether to give 4.0 g (28% yield) of IIIc, mp 118–122°. A second crop of 3.7 g (26% yield) was collected by addition of 400 ml of ether to the mother liquor. Recrystallization from propanol-ether gave an analytical sample of IIIc as a dark-blue amorphous powder, mp 120–125°; UV: λ_{max} (0.1 N HCl) 636 (ϵ 9500), 593 (10,300), and 240 (23,800) nm; λ_{sh} (0.1 N HCl) 550 (ϵ 6200) and 270 (11,900) nm.

Anal.—Calc. for $\text{C}_{22}\text{H}_{28}\text{N}_4\text{O}_5$: C, 61.67; H, 6.59; N, 13.08. Found: C, 61.65; H, 6.69; N, 13.09.

Biological Methods—Chinese hamster ovary cells² were grown in a

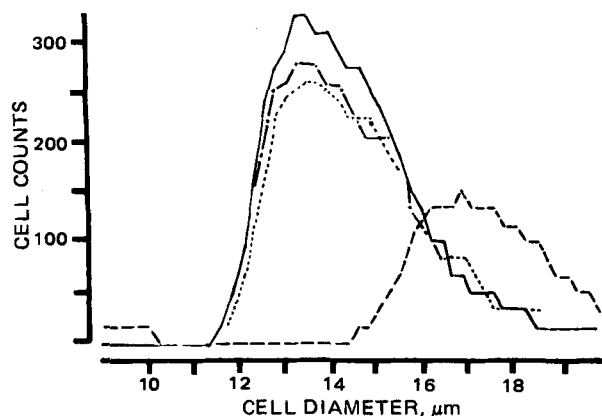


Figure 2—Cell counts and cell-sizing data for II. Every point of each line represents the average value of II on triplicate cultures. Key: —, control; --, 1000 nM; ····, 200 nM; and - · - ·, 10 nM.

² Supplied by Dr. Don Roufa, Kansas State University, Manhattan, Kans.

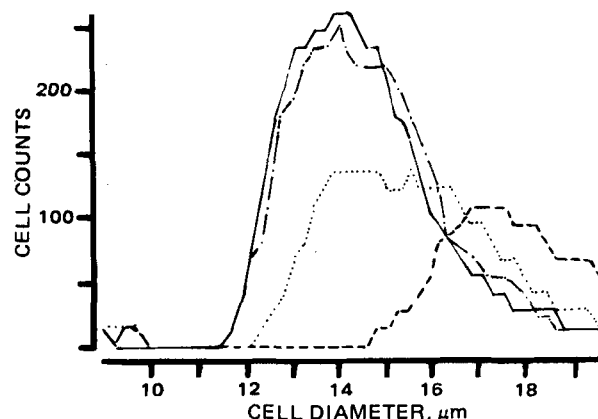
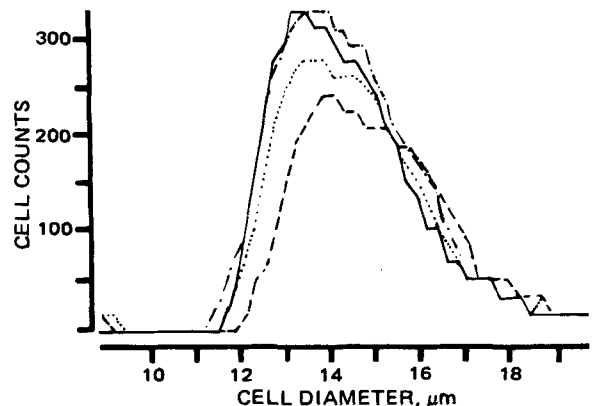
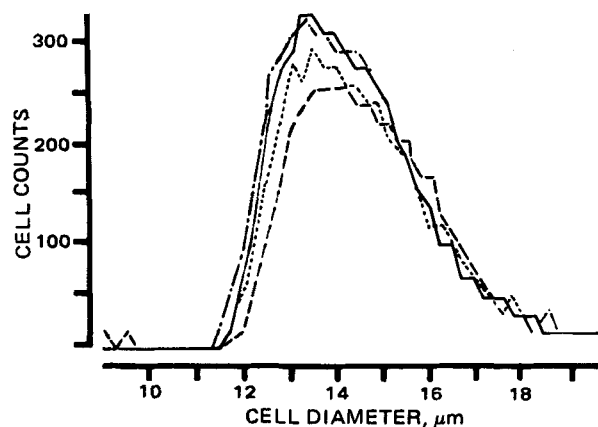


Figure 3—Cell counts and cell-sizing data for IIIa (top), IIIb (middle), and IIIc (bottom). Every point of each line represents the average value of triplicate cultures. Key: —, control; --, 1000 nM; ····, 200 nM; and - · - ·, 10 nM.

medium³ supplemented with 10% newborn calf serum⁴, selenium (30 nM), and insulin (10 ng/ml) and contained twice the glucose concentration (4 g/liter).

Cells were cultured at a concentration of $1-2 \times 10^5$ cells/dish in 1 ml. All drug concentrations were in triplicate cultures. After overnight incubation, drugs were added and cultured for an additional 24 hr. Cells were trypsinized, diluted with the counting fluid, and analyzed with electronic cell-counting and cell-sizing equipment⁵.

Data from 100 channels were stored on microprocessor disks and analyzed. Each line of the graphs (Figs. 1–5) represents the average of values obtained from triplicate cultures. The ordinate is expressed as the number of cell counts. The data from the cell-sizing equipment were calculated so that the abscissa represents cell diameter in micrometers. Hence, the

³ Roswell Park Memorial Institute 1640; obtained from KC Biological, Lenexa, Kans.

⁴ KC Biological, Lenexa, Kans.

⁵ Isoton solution, a ZBI cell counter, and a 100-channel Channelyzer were obtained from Coulter Electronics, Hialeah, Fla.

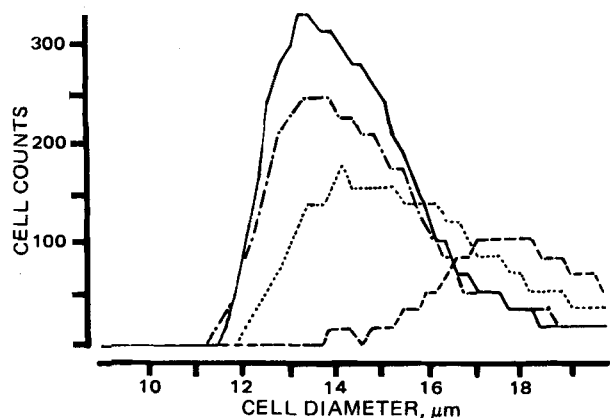


Figure 4—Cell counts and cell-sizing data for IV. Every point of each line represents the average value of IV on triplicate cultures. Key: —, control; --, 1000 nM; ····, 200 nM; and - · - ·, 10 nM.

data for each graph are given in terms of the number of cells for a given cell diameter.

RESULTS

Cell counts and cell-sizing data for I and doxorubicin (II) are shown in Figs. 1 and 2, respectively. At 1000 nM (1×10^{-6} M), both I and II inhibited cell proliferation and shifted the population toward larger cells. In addition, I inhibited cell replication in concentrations as low as 10 nM, whereas II did not. However, at 10 nM I, the size distribution indicated a return to cells of a smaller diameter. Moreover, 10 nM I (72% of controls) was significantly lower than 200, 50, and 10 nM II, using Duncan's new multiple-range test at the 0.01 level of significance.

Compounds IIIc (Fig. 3) and IV (Fig. 4) showed activity comparable to II; i.e., they were inhibitory at concentrations of 50 and 200 nM but not at 10 nM. At 200 and 1000 nM, IIIc and IV demonstrated that cell-size distributions shifted to a larger diameter.

Under similar culture conditions, V (Fig. 5), IIIa, and IIIb were much less effective, showing no statistically significant inhibition of cell proliferation compared with controls (Table I).

DISCUSSION

Although both I and IIIa were reported to possess an inhibitory effect on cell survival and cell cycle progression in cultured mammalian cells (12–14), I appeared to be 200 times more potent than IIIa *in vitro* (12). Consequently, I and related aminoanthraquinones, as well as doxorubicin (II), were studied at concentrations between 1000 and 10 nM and the following results were obtained:

1. Compound I was significantly inhibitory to Chinese hamster ovary cell proliferation at concentrations as low as 10 nM I (Fig. 1).
2. Doxorubicin exhibited inhibitory activity as low as 50 nM (Fig. 2).
3. The other ring-dihydroxylated bis(aminoalkylamino)-substituted

Table I—Antiproliferative Activity of Doxorubicin (II) and Analogs against Chinese Hamster Ovary Cells

Compound	Concentration, nM	Total Volume ^a × 10 ⁶ μm ³	Percent of Controls ^b
Control		10.60 ± 0.36 (3)	
I	1000	2.02 ± 0.74 (3)	19*
	200	2.55 ± 0.14 (3)	24*
	50	2.55 ± 0.11 (3)	24*
	10	7.67 ± 0.29 (3)	72*
II	1000	2.61 ± 0.11 (3)	24*
	200	9.13 ± 0.11 (3)	86*
	50	9.01 ± 0.21 (3)	85*
	10	9.48 ± 0.38 (3)	89
IIIa	1000	9.40 ± 0.34 (3)	88
	200	9.79 ± 0.90 (3)	92
	50	9.95 ± 0.22 (3)	93
	10	10.78 ± 0.17 (3)	101
IIIb	1000	8.52 ± 0.77 (3)	80
	200	9.82 ± 0.50 (3)	92
	50	11.25 ± 0.52 (3)	106
	10	11.24 ± 0.24 (3)	106
IV	1000	1.46 ± 0.12 (3)	13*
	200	6.22 ± 0.61 (3)	58*
	50	9.27 ± 0.67 (3)	87
	10	8.50 ± 0.15 (3)	80
V	1000	9.53 ± 0.52 (3)	89
	200	10.12 ± 0.32 (3)	95
	50	10.63 ± 0.22 (3)	100
	10	10.52 ± 0.24 (3)	100
Control		8.46 ± 0.26 (3)	
IIIc	1000	1.35 ± 0.15 (3)	15*
	200	5.11 ± 0.10 (3)	60*
	50	7.17 ± 0.33 (3)	84*
	10	8.25 ± 0.19 (2)	97

^a Represents the area under the curve between 11.5 and 17.0 μm in cell diameter; >95% of the cells from control cultures are included within these boundaries. Values are expressed as mean ± SE (number of determinations). ^b Values were analyzed, using analysis of variance and Duncan's new multiple-range test for significance. Asterisk represents differences between drug treated and controls at the 0.01 level of significance.

anthraquinone (IV) also was active at that concentration, but the magnitude of inhibition was not like that of I (Fig. 4).

4. The ring-monohydroxylated bis(aminoalkylamino)-substituted anthraquinone (IIIc) was active at 200 nM (Fig. 3).

5. Compound IIIb showed borderline activity at 1000 nM (Fig. 5).

6. Compounds IIIa and V (Fig. 5) were ineffective at the highest concentration (1000 nM) tested.

The importance of the N–O–O triangular pharmacophore to the antiproliferative activity of compounds of this type on Chinese hamster ovary cells was revealed since, theoretically, both I and IV possess two N–O–O sets, doxorubicin and IIIc possess one N–O–O set, and none of the other compounds contains this triangulation. The fact that IIIb showed borderline activity could be interpreted as due either to the presence of another electronegative element at the strategic position or to a gradual and incomplete replacement of the amino by a hydroxyl function under experimental conditions.

Most of the compounds studied showed good antineoplastic activity *in vivo*. Judging from the reports (3, 4, 7) that the dosage required for the unhydroxylated anthraquinones, such as IIIa, is at least 10 times higher than that required for the corresponding dihydroxyl analog, such as I, the possibility of *in vivo* hydroxylation to "complete" the N–O–O triangulation cannot be disregarded. In fact, chemical hydroxylation of 1,4-diamino-9,10-anthracenedione diboroacetate with an oxidizing agent such as sodium persulfate yielded the corresponding 5,8-dihydroxyl derivative (15), which further indicated that positions 5 and 8 are electronically preferred for hydroxylation.

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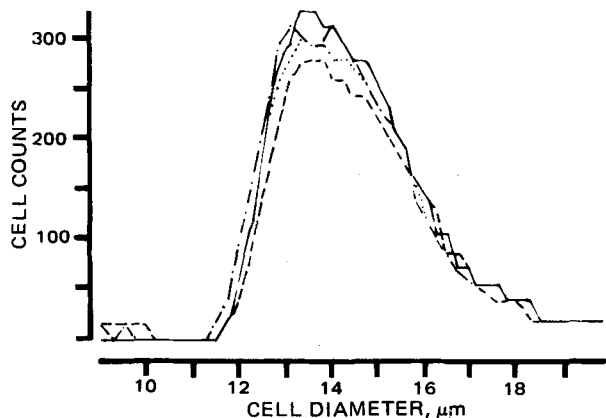


Figure 5—Cell counts and cell-sizing data for V. Every point of each line represents the average value of V on triplicate cultures. Key: —, control; --, 1000 nM; ····, 200 nM; and - · - ·, 10 nM.

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Simultaneous Determination of Vitamins B₁, B₂, B₆, and Niacinamide in Multivitamin Pharmaceutical Preparations by Paired-Ion Reversed-Phase High-Pressure Liquid Chromatography

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Abstract □ A high-pressure liquid chromatographic procedure for the simultaneous determination of vitamins B₁, B₂, B₆, and niacinamide in multivitamin pharmaceutical preparations was developed and evaluated. The method uses paired-ion reversed-phase partition chromatography for baseline separation of the four water-soluble vitamins. This method was applied to the analysis of a multivitamin and multivitamin-multimineral tablets, and a technique was developed to reduce vitamin adsorption by the minerals. The results obtained by this method were compared with those obtained by the official methods. It was concluded that this method is fast, accurate, specific, and suitable for routine quality control use.

Keyphrases □ Vitamins, water soluble—simultaneous high-pressure liquid chromatographic analyses in multivitamin preparations □ High-pressure liquid chromatography—simultaneous analyses of various water-soluble vitamins in multivitamin preparations □ Multivitamin preparations—simultaneous high-pressure liquid chromatographic assay of various water-soluble vitamins

Progress in vitamin preparations is being impeded by methodological problems. Current official methods (1, 2) for the assay of water-soluble vitamins involve a complicated sample workup, tend to reproduce poorly because of the instability and pH sensitivity of the color development, and require that each vitamin be analyzed individually. These methods also are subject to interferences from various sources when analyzing samples with complex matrixes (3, 4).

High-pressure liquid chromatographic (HPLC) procedures for the simultaneous determination of water-soluble vitamins in pharmaceutical preparations have been described (5–7), but no mechanism has been developed to control vitamin adsorption by the minerals in multivitamin-multimineral tablets. This paper describes an HPLC procedure for the simultaneous determination of vitamins B₁, B₂, B₆, and niacinamide in multivitamin and

multivitamin-multimineral tablets by paired-ion reversed-phase HPLC.

EXPERIMENTAL

Apparatus—The HPLC system included two solvent pumps¹, a solvent programmer², a UV absorbance detector³ at 280 nm, and an autoinjector⁴. A μ Bondapak phenyl (10 μ m) 30-cm \times 3.9-mm i.d. column and a 10-mv full-scale recorder⁵ were used. Peak areas were determined using a laboratory data system⁶. The column temperature was regulated at 30° by a constant temperature bath⁷. A high-speed centrifuge was used⁸, and the samples were disintegrated in a shaker bath⁹ set at 60°.

Materials and Reagents—Thiamine hydrochloride, riboflavin, pyridoxine hydrochloride, and niacinamide were obtained as USP reference standards. 1-Hexanesulfonic acid was used as received¹⁰. Anhydrous citric acid¹¹ was obtained commercially and used without further purification. Solvents, all distilled-in-glass¹² grade, were obtained commercially. Commercial multivitamin preparations were obtained from local pharmacies.

Preparation of Mobile Phases—The two pumps used 0.0025 M 1-hexanesulfonic acid in water and in methanol, respectively. Prior to use, the mobile phases were degassed by vacuum filtration through a 0.5- μ m pore, 47-mm diameter filter¹³. With a linear solvent program¹⁴, a gradient was run at a constant flow rate of 2.0 ml/min for 18 min. Solvent conditions varied from 0 to 80% methanol in water. Five minutes was allowed between sample injections.

¹ Model 6000A, Waters Associates, Milford Mass.

² Model 660, Waters Associates, Milford, Mass.

³ Model 440, Waters Associates, Milford Mass.

⁴ WISP model 710A, Waters Associates, Milford Mass.

⁵ Omniscribe, Houston Instrument, Houston, Tex.

⁶ Model 3353, Hewlett-Packard, Palo Alto, Calif.

⁷ FK2, Haak Inc., Saddle Brook, N.J.

⁸ Sorvall RC-5B centrifuge fitted with SS-34 motor, DuPont Instruments, Wilmington, Del.

⁹ Magni-Whirl water bath 15-453-400, Fisher Scientific Co., San Francisco, Calif.

¹⁰ B-6 PIC reagent, Waters Associates, Milford Mass.

¹¹ Matheson, Coleman and Bell, Norwood, Ohio.

¹² Burdick & Jackson, Muskegon, Mich.

¹³ Type LS, 47 mm, Millipore Corp. Bedford, Mass.

¹⁴ Curve 6 on model 660 solvent programmer.