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Note

High-performance liquid chromatography of cancer chemotherapeutic agents: bis(substituted aminoalkylamino)anthraquinones

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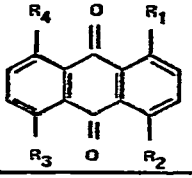
The bis(substituted aminoalkylamino)anthraquinones, hereafter referred to as the aminoanthraquinones, are a new class of cancer chemotherapeutic agents developed in response to structure-activity studies among DNA intercalating agents[1,2]. The structures of these compounds (Fig. 1) are exemplified by the parent compound of the series, 1,4-bis({2-[(2-hydroxyethyl)amino]ethyl}amino)-9,10-anthracenedione (NSC 287513). This compound and a number of the other aminoanthraquinones have been found to have very high activity against murine tumors including P388 and L1210 leukemias, B16 melanoma and colon carcinoma 26 (ref. 3). Recent studies in this laboratory have also shown that the *in vivo* active aminoanthraquinones exhibit saturable binding and specificity to a number of macromolecular lipids extracted from murine L1210 tumors[4]. It was during these latter studies that we found it necessary to develop methods to assess the purity of the various aminoanthroquinones prior to their use in binding and kinetic experiments. We thus report here the successful separation of a number of aminoanthraquinones by high-performance liquid chromatography (HPLC). Except for a preliminary report on the purification of NSC 287513 using a μBondapak C_{18} reversed-phase system[5], this is the first report of the HPLC analysis of these compounds.

EXPERIMENTAL

Chemicals

Analytical-reagent HPLC grade solvents were used throughout this study after appropriate micropore filtration and degassing. Water was first deionized and then glass-redistilled prior to use. Commercially available HPLC grade water was found unsuitable for use on reversed-phase columns, resulting in spurious peaks when used in acetonitrile and especially methanol gradients. Anthraquinone, 1,4-diaminoanthraquinone, 1,5-diaminoanthraquinone, 1-amino-4-hydroxy-anthraquinone and 1,4-dihydroxy-anthraquinone were purchased from Aldrich (Milwaukee, Wisc., U.S.A.). All bis(substituted aminoalkylamino)anthraquinones were provided by Drug Research and Development, Division of Cancer Treatment, National Cancer Institute, Bethesda, Md., U.S.A. All buffer salts were analytical-reagent grade.



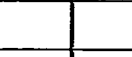
Compound	R_1	R_2	R_3	R_4
Anthraquinone	–H	–H	–H	–H
1,4-Diaminoanthraquinone	$–NH_2$	$–NH_2$	–H	–H
1,5-Diaminoanthraquinone	$–NH_2$	–H	$–NH_2$	–H
1-Amino-4-hydroxy-anthraquinone	$–NH_2$	–OH	–H	–H
1,4-Dihydroxyanthraquinone	–OH	–OH	–H	–H
NSC 287513	$–NH(CH_2)_2NH(CH_2)_2OH$	same as R_1	–H	–H
NSC 276740	$–NH(CH_2)_2NHCH_2CH_3$	same as R_1	–H	–H
NSC 278467	$–NH(CH_2)_3NH(CH_2)_2OH$	same as R_1	–H	–H
NSC 279836	$–NH(CH_2)_2NH(CH_2)_2OH$	same as R_1	–OH	–OH
NSC 279837		same as R_1	–H	–H
NSC 279838		same as R_1	–H	–H
NSC 281246	$–NH(CH_2)_2NH_2$	same as R_1	–H	–H
NSC 281249	$–NH(CH_2)_2NH(CH_2)_2NH(CH_2)_2OH$	same as R_1	–H	–H
NSC 285688	$–NH(CH_2)_2S(CH_2)_2OH$	same as R_1	–H	–H
NSC 291923	$–NH(CH_2)_2NHCH_3$	same as R_1	–H	–H
NSC 291924	$–NH(CH_2)_2NH(CH_2)_2CH_3$	same as R_1	–H	–H
NSC 293844	$–NH(CH_2)_3N(CH_3)_2$	–H	same as R_1	–H
NSC 294725	$–NH(CH_2)_3N(CH_3)_2$	–H	–H	same as R_1
NSC 294726	$–NH(CH_2)_3N(CH_3)_2$	same as R_1	–H	–H
NSC 295560	$–S(CH_2)_2N(CH_3)_2$	–H	same as R_1	–H
NSC 295561	$–NH(CH_2)_2N(CH_3)_2$	–H	same as R_1	–H
NSC 299187	$–NH(CH_2)_2NH(CH_2)_2OH$	–OH	–H	–H
NSC 300576	$–NH(CH_2)_2N(CH_3)_2$	same as R_1	–H	–H
NSC 300579	$–NH(CH_2)_2N(CH_3)_2$	–OH	–H	–H
NSC 300580	$–O(CH_2)_2N(CH_3)_2$	–H	–H	–H

Fig. 1. Structures of the anthraquinones and bis(substituted aminoalkylamino)anthraquinones analyzed in this study.

Apparatus

A Micromeritics Model 701-21204-02 liquid chromatograph (Micromeritics, Norcross, Ga., U.S.A.) equipped with full gradient elution capacity, a variable wavelength ultraviolet–visible detector and a column oven was used in these studies. In addition, the eluate from the detector was passed through the flow cell of a Varian Model 635 ultraviolet–visible scanning spectrophotometer (Varian, Palo Alto, Calif., U.S.A.) which was used with the stop-flow feature available on the Micromeritics instrument to determine the absorbance spectrum of the column eluate at any point during an analysis. The column eluate was finally directed to a fraction collector allowing collection of the separated aminoanthraquinones for further analysis. If required, most of the aminoanthraquinones could be extracted from the HPLC column effluents with methylene chloride, ethyl acetate or chloroform.

Detection of the aminoanthraquinones in the column eluates was routinely

carried out with the HPLC detector at 254 nm. When scanning was not required, the eluates were also monitored at 630 nm with the spectrophotometer, thus providing a dual wavelength monitoring capability.

The columns used, μPorasil, μBondapak C_{18}, μBondapak CN and μBondapak NH_2 (Waters Assoc., Milford, Mass., U.S.A.), were 30 cm × 4 mm I.D. in size. The columns were routinely assessed for theoretical plate counts and efficiency according to the manufacturer's instructions and were discarded if changes in these parameters occurred. Column flow-rate was routinely 2.0 ml/min and operating pressures ranged from 1500 to 2800 p.s.i.

Systems

A number of solvent and gradient systems utilizing a variety of aqueous buffer salt solutions in combination with methanol, acetonitrile and 2-propanol were used in development of the reported methods. The two most efficient systems used were as follows:

(1) μBondapak CN column, eluting with a 15-min convex mobile phase gradient (curvature = 4) of 20–90% methanol in 0.05 *M* Na_2HPO_4–NaH_2PO_4, pH 8.0, and a flow-rate of 2.0 ml/min.

(2) μBondapak NH_2 column, eluting with a 15-min convex mobile phase gradient (curvature = 4) of 10–90% acetonitrile–methanol (1:1) in 0.05 *M* ammonium acetate–ammonium hydroxide pH 8.0 and a flow-rate of 2.0 ml/min.

RESULTS AND DISCUSSION

Initial trials on aminoanthraquinone separations using normal phase columns such as μPorasil and solvent combinations of chloroform with various alcohols resulted in the compounds being either retained on the column (retention time >90 min) or eluted as broad and/or skewed peaks. Thus, normal-phase HPLC of the aminoanthraquinones was abandoned and reversed-phase columns were evaluated. Initially, μBondapak C_{18} columns were used, eluting with aqueous mixtures in methanol and acetonitrile. While the aminoanthraquinones did elute from this column, such elution was characterized by long retention times and poor resolution of mixtures. The μBondapak CN and μBondapak NH_2 columns were next evaluated and both proved suitable for aminoanthraquinone analysis. Replacement of the water in the mobile phase with various buffer salt solutions markedly enhanced elution and resolution of the compounds. This was especially apparent with the μBondapak CN column which required the relatively strong buffering capacity of sodium phosphate for aminoanthraquinone elution. Variation of the mobile-phase aqueous solution pH from pH 4.0 to 8.0 resulted in maximum resolution of the aminoanthraquinones at pH 8.0. More basic solutions were not used since it has been reported that exposure of silica based reversed-phase packing materials to basic solvents (pH > 7.5) results in accelerated degradation of the packing material[6]. Variations in gradient times from 10 to 40 min and in gradient curvature utilizing linear, convex and concave curvatures established that a gradient time of 15–25 min together with a convex gradient curvature of from 2 to 4 was optimal for aminoanthraquinone separations on either column. Initial trials utilizing the μBondapak NH_2 column showed that the use of methanol in the mobile-phase resulted in very long aminoanthraquinone retention

times while the use of acetonitrile resulted in shorter retention times but loss of mixture resolution. Thus, mixtures of acetonitrile–methanol were tested and found, at a 1:1 volume ratio, to optimalize resolution of the aminoanthraquinones on the μBondapak NH_2 column.

Table I lists the retention times found for the anthraquinones and aminoanthraquinones studied utilizing the μBondapak CN and μBondapak NH_2 columns in systems 1 and 2, respectively. Fig. 2 illustrates the separation of nine aminoanthraquinones in system 1. The separation shown was carried out at a column oven temperature of 50°. Comparison of aminoanthraquinone separations on system 1 at 22, 25, 30, 40, 50 and 60° established that the separations were significantly enhanced at the higher temperatures with concurrent decreases in aminoanthraquinone retention times, especially in the cases of the more non-polar compounds such as NSC 294725, 294726 and 279837. In fact, HPLC of the latter compounds at 25° in system 1 resulted in broad and skewed elution peaks with elution times >30 min. A similar enhancement of aminoanthraquinone separation was observed at elevated temperatures with the μBondapak NH_2 column (system 2) as shown in Table I which com-

TABLE I

RETENTION TIMES OF THE BIS(SUBSTITUTED AMINOALKYLAMINO)ANTHRAQUINONES AND RELATED COMPOUNDS

Compound	*Retention time (min)*		
	*System 1**	*System 2**	
		25°	*50°*
Anthraquinone	7.1	3.9	3.6
1,4-Diaminoanthraquinone	4.7	2.6	2.7
1,5-Diaminoanthraquinone	4.1	2.8	2.9
1-Amino-4-hydroxy-anthraquinone	5.4	3.8	3.6
1,4-Dihydroxy-anthraquinone	6.4	3.9	3.8
NSC 287513	7.3	6.8	6.3
NSC 276740	14.9	12.6	11.4
NSC 278467	13.6	7.3	7.1
NSC 279836	8.3	9.3 (B)**	8.0
NSC 279837	32.0	52.2	50.9
NSC 279838	9.7	9.4	9.1
NSC 281246	7.3	10.3	9.4
NSC 281249	6.0	4.7	4.7
NSC 285688	4.7	3.7	3.5
NSC 291923	10.0	5.7	5.1
NSC 291924	11.7	9.7	8.4
NSC 293844	13.8	12.3	11.2
NSC 294725	18.5	38.9 (B)	32.4
NSC 294726	19.7	57.8 (B)	41.1
NSC 295560	8.9	6.8	7.1
NSC 295561	9.4	14.5	12.4
NSC 299187	6.3	6.8	6.3
NSC 300576	10.8	11.1	9.6
NSC 300579	6.7	5.2	5.3
NSC 300580	6.4	3.7; 4.3	4.1; 4.4

* See the text for descriptions of each system.

** B denotes a broad and usually unsymmetrical elution peak.

pares the elution times of the compounds studied in system 2 at 25 and 50°. In addition, Fig. 3 illustrates the separation of eight aminoanthraquinones in system 2 at 25 and 50°. Most notable in the latter comparison is the decreased total time required for the separation from 23 to 18 min, and the increased resolution of the mixture including resolution of NSC 287513 and 278467 at 50°. Our results agree with those of other workers who have reported increased resolution and decreased retention times utilizing reversed-phase columns and increased temperatures[7,8]. Thus, elevated temperature appears a valuable aid in the HPLC analysis of the aminoanthraquinones. We chose not to exceed 50° in our final systems since trials at 60° did not appreciably increase the resolution of the aminoanthraquinone mixtures and since we wished to limit any thermal stress on the columns.

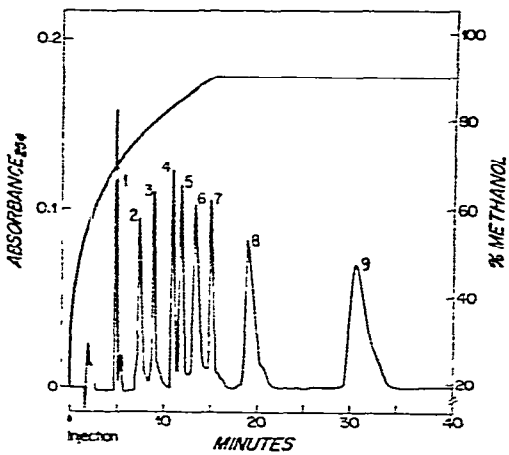


Fig. 2. Separation of nine aminoanthraquinones on system 1 at 50°. Sample amounts were 1–2 μg of each compound. Attenuation was 0.2 absorbance units full scale. 1, NSC 285688; 2, NSC 287513; 3, NSC 295561; 4, NSC 300576; 5, NSC 291924; 6, NSC 278467; 7, NSC 276740; 8, NSC 294725; 9, NSC 279837.

The value of the on-line scanning spectrophotometer in our system is illustrated by the case of compound NSC 293844. As supplied to us, the compound is resolved into two peaks in both HPLC systems with a peak area ratio between peaks 1 and 2 of 2:3. In system 2 at 50°, peaks 1 and 2 have respective retention times of 5.3 and 11.2 min. While both peaks are detected at 254 nm, niether is detected at 630 nm, an observation not unexpected since the compound has a visible absorbance maximum in methanol–water (1:1) of 526 nm[9]. By utilizing the stop-flow mode on the HPLC and scanning each peak from 250–700 nm, it was found that peak 2 had the expected absorbance spectrum for NSC 293844, while peak 1 had no visible absorbance maxima. Thus, peak 1 was assumed to represent a contaminant of NSC 293844. It was also found that NSC 300580 was resolved into two major peaks upon HPLC analysis (Fig. 3). Spectrophotometric scans of each peak resulted in very similar spectra, each with the expected visible absorbance maximum at 374 nm for the compound[9]. We thus concluded that the two peaks represent isomers of the compound although no attempt was made to define the structures of the two compounds.

The HPLC systems described are capable of resolving positional isomers present among the aminoanthraquinones analyzed. For example, the aminoanthra-

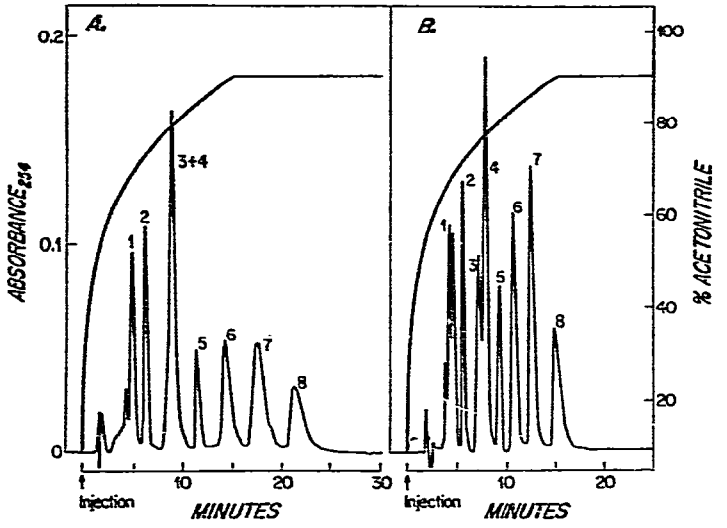


Fig. 3. Separation of eight aminoanthraquinones on system 2 at (A) 25° and (B) 50°. Sample amounts and attenuation were as described in Fig. 2. 1, NSC 300580; 2, NSC 291923; 3, NSC 287513; 4, NSC 278467; 5, NSC 291924; 6, NSC 300576; 7, NSC 276740; 8, NSC 295561.

quinones NSC 294726, 293844 and 294725 represent the 1,4-, 1,5- and 1,8-dimethylaminopropylamino isomers of the same aminoanthraquinone. All three compounds are well resolved in both HPLC systems (Table I) in the retention time sequence of 1,5 < 1,8 < 1,4.

The successful development of the reported methods for aminoanthraquinone separation represents a valuable analysis aid not only for purity determinations but also for the separation of related new compounds with potential chemotherapeutic use. In addition, the described methods are applicable to preclinical and clinical studies directed at the physiological disposition of aminoanthraquinones in biological samples.

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