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Note

Separation and detection of 2-aminoanthracene and its metabolites by high-performance liquid chromatography

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Recently, the carcinogen 2-aminoanthracene (2-AA) has been recommended for use as a positive control in the Salmonella/microsome mutagenicity assay since it causes mutations in all tester strains[1]. The metabolism of 2-AA to mutagenic products is also enhanced in the presence of lithocholic acid and its derivatives[2] and other modifiers of drug metabolism[3]. For metabolism studies with this carcinogen it is important to have an analytical method capable of detecting the compound and its metabolites. However, these metabolic products, which heretofore have been unavailable, are unstable and no chromatographic method for their detection has been reported. By derivatization of 2-AA and its metabolites to stable products, we can now separate them by high-performance liquid chromatography (HPLC). The application of the technique to some biological studies was examined.

EXPERIMENTAL

Reference standards and reagents

2-AA was obtained from Aldrich (Milwaukee, WI, U.S.A.). 2-Acetylaminoanthracene, 2-acetylamino-9,10-anthraquinone, 2-nitroanthracene, 2-nitroanthraquinone, N-hydroxy-2-aminoanthracene, and the monoacetoxy derivatives of the 5-, 6-, and 8-hydroxy-2-acetylaminoanthracenes were synthesized in our laboratory[4]. Glass-distilled organic solvents (Burdick & Jackson Labs., Muskegon, MI, U.S.A.) were used. All other reagents were of the highest purity commercially available.

Preparation of microsomes and 2-AA metabolism samples

Male Sprague-Dawley rats (up to 250 g) were obtained from the National Cancer Institute Animal Facility in Bethesda, MD. The animals were housed in plastic shoe-box cages, and given water and Purina chow *ad libitum*. Liver 9000 *g* supernatant fractions (S9) were prepared from animals pretreated (IP) with Aroclor 1254 (one 500 mg/kg dose dissolved in corn oil 5 days before sacrifice). Liver S9 fractions were prepared as described previously[2].

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2-AA and its metabolites were assayed in the presence of the same concentrations of S9 protein which were present when this compound was tested in the Ames assay[3], *i.e.* 1 μg of 2-AA per 0.5 mg of AC-S9 protein. The assay mixture also contained the following components in a total volume of 1 ml: 100 μmoles potassium phosphate, pH 7.4, 3 μmoles $MgCl_2$, 5 μmoles glucose-6-phosphate, 1 unit of glucose-6-phosphate dehydrogenase, 400 μmoles NADPH, and varying amounts of liver S9 fraction. The reactions were stopped by the addition of 1 ml of acetone and stored on ice; 2 ml of ethyl acetate were added and the solutions vortexed vigorously for 30 sec. After centrifugation, the organic phase was removed, dried over sodium sulfate and then acetylated with acetic anhydride (100 μl per 2.5 ml for 15 min at room temperature). The solutions were evaporated to dryness under N_2 and stored at −20°C. When analyzed as methylurea derivatives, the reactions were stopped by extraction with 5 ml of methylene chloride. The extracts were treated with 0.2 ml of methyl isocyanate and warmed at 37°C for 15 min prior to evaporation to dryness under N_2. The dried extracts were stored at −20°C.

Preparation of methylurea standards

Approximately 1 mg each of 2-AA and N-hydroxy-2-AA were dissolved in 3 ml of methylene chloride. Methyl isocyanate (0.3 ml) was added and after standing at room temperature for 2 h the solutions were evaporated to dryness under N_2. The residue was dissolved in a minimal volume of methanol and applied to a 0.25-mm silica gel TLC plate (20 × 20 cm, Analtech Rediplate). They were developed with isopropanol–hexane (5:95, v/v). After the plates were developed, the bands were visualized under long-wavelength UV light. The methylurea derivative of 2-AA migrated as a bright blue band. The band was scraped off the plate and eluted with acetone. The N-hydroxy-2-AA derivative separated into two major bands which appeared blue under the UV light. One of them had the same R_F as the 2-AA derivative. The other was more polar as might be expected of the methylurea derivative of N-hydroxy-2-AA. The N-hydroxy-2-AA was contaminated with, at least, 25% 2-AA. Infrared analysis of the isolated fraction indicated the presence of methylurea derivatives.

High-performance liquid chromatography

A modular HPLC system was used for this study. The unit consisted of a Laboratory Data Control (Riviera Beach, FL, U.S.A.) Constametric I and II pumps, a Gradient Master and Mixer; a Chromatronix dual-channel UV absorbance detector (Spectra-Physics, Santa Clara, CA, U.S.A.); a Rheodyne Model 7120 syringe-loading sample injector (Rheodyne, Berkeley, CA, U.S.A.); and a Fisher Recordall Series 5000 recorder (Fisher Scientific, Silver Spring, MD, U.S.A.) operated at 0.2 in/min chart speed and a 10 mV setting.

Separations were made on a 25 cm × 4.6 mm I.D. 10 μ-Partisil column (Whatman, Clifton, NJ, U.S.A.) operated at ambient temperature and a flow-rate of 1 ml/min of mobile phase 1–10% isopropanol in hexane with a linear gradient of 15 min followed by 45 min under the final conditions. An isocratic mobile phase of ethanol–heptane (20:80 v/v), was used for the separation of methylurea derivatives. A precolumn (4 cm × 2.1 mm I.D.) packed with Waters Assoc. (Milford, MA, U.S.A.) pellicular Corasil (37–50 μm) was used to ensure the stability of the analytical column.

Peak areas and retention times were determined with a Hewlett-Packard 3352A laboratory data system (Avondale, PA, U.S.A.). The residue obtained from metabolism samples was dissolved in 100 μl mobile phase (10% isopropanol in hexane) prior to HPLC analysis.

RESULTS AND DISCUSSION

A typical HPLC separation of 2-AA and its metabolites on a μPartisil column using a 15 min linear gradient from 1–10% propan-2-ol in hexane is shown in Fig. 1. The approximate amounts are given in the figure caption. Retention times, peak areas and peak shapes were highly reproducible under the conditions used. When an isocratic mobile phase of 10% isopropanol in hexane was used, baseline separation of all but the nitro derivatives was achieved. Table I shows the retention times of the compounds when resolved either by gradient or isocratic mobile phase conditions. Improved resolution of nitro-derivatives was obtained under gradient conditions. N-hydroxy-2-AA did not elute under these mobile phase conditions, presumably because it was strongly adsorbed to the silica gel column.

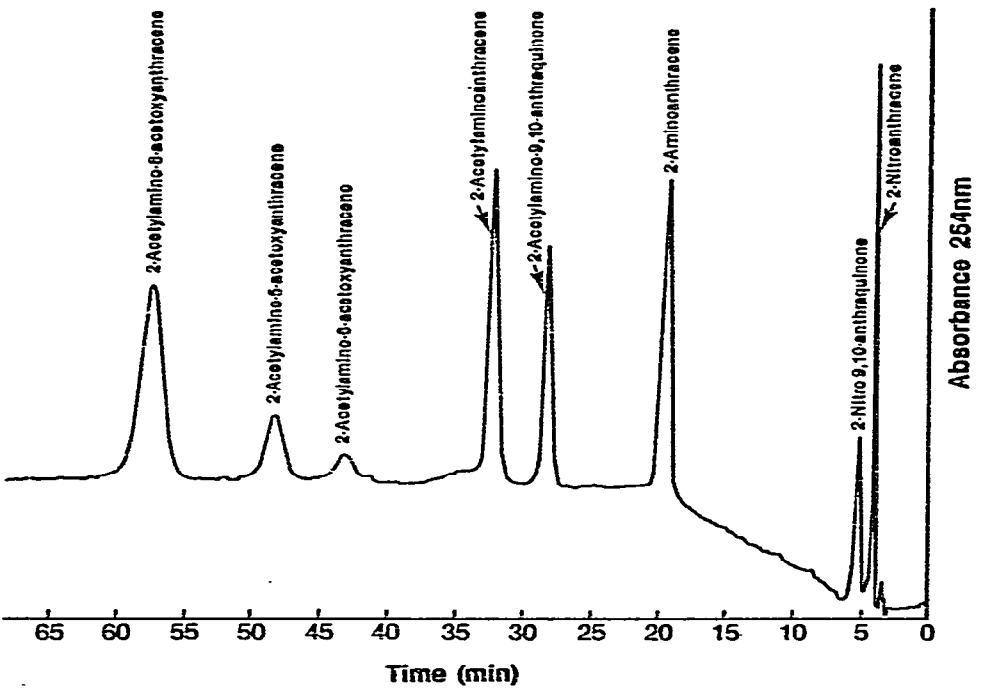

Fig. 1. HPLC profile of mixed standard. Approximate amounts are given in parentheses. 2-Nitroanthracene (45 ng), 2-nitro-9,10-anthraquinone (65 ng), 2-aminoanthracene (155 ng), 2-acetylamino-9,10-anthraquinone (300 ng), 2-acetylaminoanthracene (100 ng), 2-acetylamino-6-acetoxyanthracene (110 ng), 2-acetylamino-5-acetoxyanthracene (300 ng), and 2-acetylamino-8-acetoxyanthracene (500 ng) were separated on a 10 μ-Partisil column using a linear gradient of 1–10% isopropanol in hexane for 15 min followed by 45 min under the final conditions. UV absorbance was monitored at 254 nm with full-scale sensitivity at 0.04. Flow-rate of 1 ml/min and chart speed of 0.2 in/min were used.

TABLE I

RETENTION TIMES OF 2-AA AND METABOLITES UNDER GRADIENT AND ISOCRATIC MOBILE PHASE CONDITIONS

Compound	*1–10% Isopropanol in hexane (gradient)*	*10% Isopropanol in hexane (isocratic)*
2-Nitroanthracene	4.0	3.9
2-Nitro-9,10-anthraquinone	5.1	4.3
2-Aminoanthracene	17.6	11.4
2-Acetylamino-9,10-anthraquinone	27.7	17.9
2-Acetylaminoanthracene	31.7	22.7
2-Acetylamino-6-acetoxyanthracene	42.4	35.8
2-Acetylamino-5-acetoxyanthracene	47.3	41.7
2-Acetylamino-8-acetoxyanthracene	56.4	52.3

Metabolites of 2-AA produced by incubation with liver S9 fractions from rats pretreated with Aroclor 1254 were derivatized for the HPLC analysis. The results obtained after incubation of 1 μg of 2-AA per ml for 0, 5, and 15 min with 10 μl (0.25 mg/ml) S9 fractions showed that no derivatives of 2-AA were present. However, the disappearance of 2-AA both as a function of S9 protein concentration and time is shown in Fig. 2.

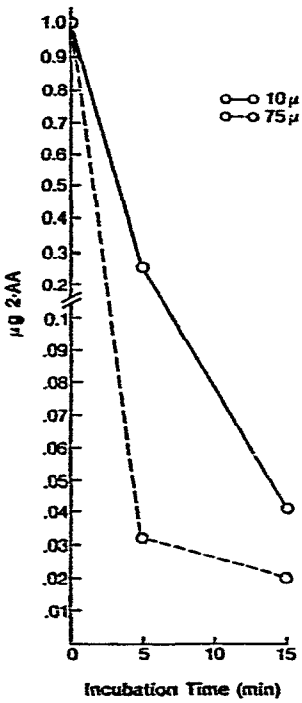

Fig. 2. Plot of disappearance of 2-AA as a function of time and protein concentration. 1 μg of 2-AA was incubated for 0, 5 and 15 min with 10 μl (O——O) and 75 μl (O– – –O) S-9 fractions.

To facilitate the elution of N-hydroxy-2-AA from the silica gel column, its methylurea (MU) derivative and also that of 2-AA were prepared and purified by TLC (see Experimental). With a stronger mobile phase of 20% isopropanol in hexane (isocratic) the N-hydroxy-2-AA-MU eluted at *ca.* 33 min as a broad peak, whereas 2-AA-MU eluted at 13 min as a sharp peak. When 20% ethanol in heptane (isocratic) was used as a mobile phase, sharper peaks of both N-OH-2-AA-MU and 2-AA-MU at *ca.* 15 and 7.4 min, respectively, eluted (Fig. 3).

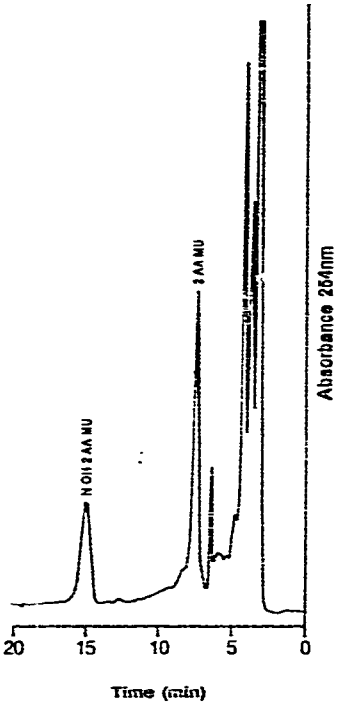

Fig. 3. HPLC profile of methylurea derivatives of 2-AA and N-hydroxy-2-AA. Conditions same as Fig. 1 with the exception of an isocratic mobile phase of ethanol–heptane (20:80 v/v).

The enthanol–heptane mobile phase was used to analyze extracts of incubations of 1 μg of 2-AA for 10 min with 0, 5, 10 and 25 μl (0, 0.12, 0.25 and 0.62 mg/ml) of S9-fraction. In Fig. 4 the increasing rate of disappearance of 2-AA with increasing amounts of S9 protein is shown. N-hydroxy-2-AA metabolite peaks, or other peaks which might correspond to phenolic metabolites, were not observed. Experiments to determine if the loss of N-hydroxy-2-AA results from non-specific binding to protein are continuing. Incubation of higher concentrations of 2-AA (10 μg/ml) also resulted in measurable losses of 2-AA, and no products eluted with expected metabolites even when 50% of the total extract was analyzed.

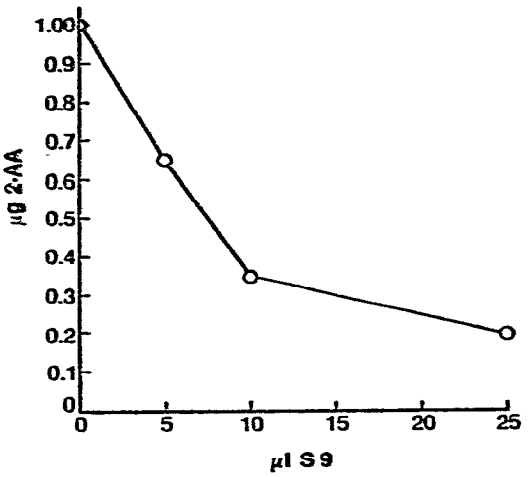

Fig. 4. Plot of disappearance of 2-AA as a function of protein concentration. 1 μg of 2-AA was incubated for 10 min with 0, 5, 10 and 25 μl S9 fractions.

CONCLUSIONS

A useful HPLC method for the separation and detection of 2-AA and its metabolites has been developed. The method was used to separate and quantitate the disappearance of 2-AA from *in vitro* incubations containing S9 fractions from rats. Even though total lipid extracts were used, the contaminating lipids did not interfere with the detection and elution of the metabolites in either mobile phase system. Although no N-hydroxy-2-AA and phenolic metabolites were detected, the sensitivity of the method could be substantially increased by using radiolabeled substrate. Hence the methods reported here have the potential for use in studies involving *in vivo* and *in vitro* studies of 2-AA metabolism.

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