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SEPARATION OF 1,2-DIMETHYLHYDRAZINE METABOLITES BY HIGH-PRESSURE LIQUID CHROMATOGRAPHY

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SUMMARY

The separation of 1,2-dimethylhydrazine, azomethane, azoxymethane, methylazoxymethanol, methylazoxymethanol acetate, formaldehyde and methanol by high-pressure liquid chromatography on columns of C_{18}/Corasil, μBondapak C_{18} and Aminex A-27 is described. The separations are highly reproducible and rapid and may be used for kinetic studies. An example of an application of these methods to the analysis of metabolites in rat urine derived from [^{14}C]-1,2-dimethylhydrazine is reported.

INTRODUCTION

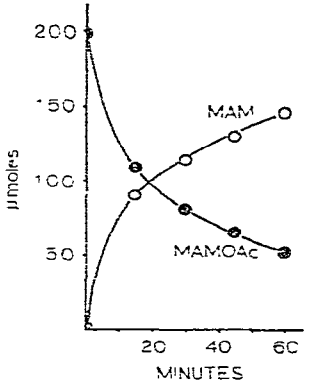
As an essential part of our attempts to clarify the metabolism and mechanism of action of 1,2-dimethylhydrazine (1,2-DMH), a highly potent and specific colon carcinogen in various rodent species[1], we have devoted considerable effort towards developing suitable methods for the separation and detection of its metabolic products. In a previous communication[2] we have described an efficient thin-layer chromatographic method for the separation of 1,2-DMH from hydrazine, monomethylhydrazine and 1,1-dimethylhydrazine, compounds which are frequently encountered as contaminating by-products during the synthesis of 1,2-DMH. In the present report, we describe methods utilizing high-pressure liquid chromatography (HPLC) to effect separations of 1,2-DMH from azomethane (CH_3–N=N–CH_3; AM), azoxymethane (CH_3–N=N–CH_3; AOM), methylazoxymethanol (CH_3–N=N–CH_2OH; MAM),

formaldehyde and methanol, compounds which have been postulated[1,3] as intermediates in the metabolic activation of 1,2-DMH.

EXPERIMENTAL

[^{14}C]-1,2-DMH, [^{14}C]-formaldehyde and [^{14}C]-methanol were obtained from New England Nuclear (Boston, Mass., U.S.A.). AOM and methylazoxymethanol

acetate (MAMOAc) were obtained from Microbiological Assoc. (Bethesda, Md., U.S.A.) through the courtesy of Dr. E. Weisburger, Carcinogenesis Programs, National Cancer Institute (Bethesda, Md., U.S.A.). MAM was prepared by the enzymatic hydrolysis of MAMOAc at pH 7.4 (Fig. 1) using purified hog liver esterase (Type II, Sigma, St. Louis, Mo., U.S.A.). Since MAM is unstable (Fig. 2), decomposing spontaneously into products, including formaldehyde, nitrogen and methanol[4], this compound was kept at 0° and used within about 2 h of its preparation. AM was prepared by the oxidation of 1,2-DMH with sodium chromate[5]. Because of its volatility (b.p. 2°) and its toxic and explosive properties, AM was not isolated but rather prepared as a solution by bubbling nitrogen through the reaction mixture and collecting the gas in ethanol cooled to −70°.



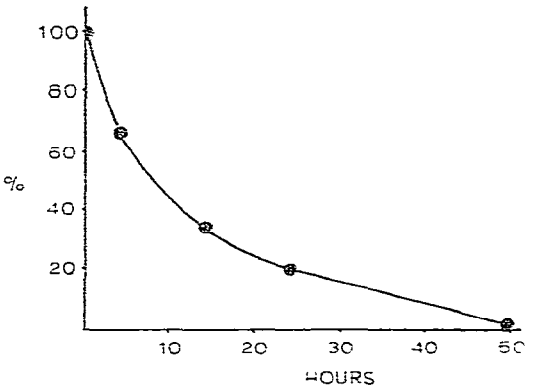


Fig. 1. Preparation of MAM from MAMOAc by enzymatic hydrolysis. 200 μmoles of MAMOAc in 1 ml of 0.1 *M* potassium phosphate buffer (pH 7.4) was incubated with 20 μl of a suspension of commercial porcine liver esterase at 37°. Aliquots (5 μl) were taken periodically and assayed using a C_{18}/Corasil column (see Fig. 3). Relative amounts of MAM and MAMOAc were determined by measuring the area under the respective peaks obtained from a recording of absorbance at 217 nm. Enzyme protein in the sample applied to the column did not interfere with the assay since it eluted in the void volume, ahead of MAM.

Fig. 2. Stability of MAM at pH 7.4 at 37°. 20 μmoles MAM, prepared by the enzymatic hydrolysis of MAMOAc and purified by HPLC on C_{18}/Corasil was incubated at 37° in 1 ml of 0.1 *M* potassium phosphate buffer (pH 7.4). Aliquots were taken periodically and assayed on C_{18}/Corasil as in Fig. 1.

A Waters Assoc. HPLC system equipped with Model 6000 pumps and a Model U6K sample loop injector was used in conjunction with a Schoeffel Model SF 770 dual-beam variable-wavelength flow analyzer. C_{18}/Corasil (37–50 μm; Waters Assoc., Milford, Mass., U.S.A.) was dry packed with mechanical vibration in 1/8 × 36 in. stainless-steel columns. μBondapak C_{18} columns (4 mm × 30 cm) were obtained prepacked from Waters Assoc. Aminex A-27 (Bio-Rad Labs., Richmond, Calif., U.S.A.), a polystyrene–divinyl benzene strong anion-exchange resin (particle size 13.5 μm) was packed in a 3/8 × 24 in. stainless-steel column using the slurry method, and converted to the acetate form prior to use. All aqueous eluents were filtered through a Millipore 0.45 μm filter. μBondapak C_{18} and C_{18}/Corasil columns were stored under acetonitrile or methanol when not in use to retard deterioration.

RESULTS AND DISCUSSION

Separation of MAM, AOM, AM and MAMOAc was effective on both C_{18}/Corasil and μBondapak C_{18} using either water or 1% ethanol as eluent (Figs. 3 and 4). A 1-% ethanol solution in water was used routinely, however, since it decreased trailing of MAMOAc. With either eluent, 1,2-DMH was strongly retained on both columns and could be eluted only when the pH of the eluent was lowered, *e.g.*, with the use of 0.05 *M* acetic acid which apparently suppresses dissociation of residual, non-substituted silanol groups on the Corasil and μBondapak silica matrix, or, alternately, when the pH was increased as with the use of 1% ammonium carbonate, to decrease the ionization of 1,2-DMH ($pK_a' = 7.52$; ref. 6). Thus both types of columns evidently function not only by partition in the reverse-phase mode, but also by virtue of ion exchange. Separations of the azo and azoxy compounds obtained with μBondapak C_{18} were superior to those obtained with C_{18}/Corasil. Thus, for ex-

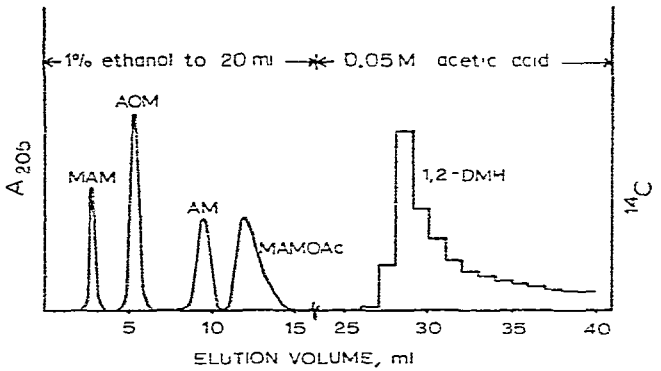


Fig. 3. Resolution of a test mixture of [^{14}C]-1,2-DMH and probable metabolites on C_{18}/Corasil. Two 1/8 × 36 in. columns connected in series were eluted with 1% ethanol (1 ml/min) for the first 20 ml to elute the first four compounds. After 20 ml the eluent was changed to 0.05 *M* acetic acid to elute [^{14}C]-1,2-DMH. Radioactivity was detected in collected 1-ml fractions by liquid scintillometry. Azo and azoxy compounds were detected by monitoring the effluent at 205 nm. Pressure, 1200 p.s.i.

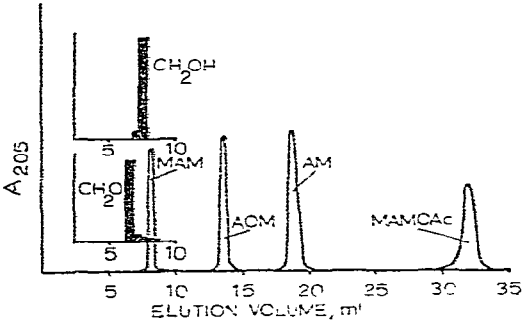


Fig. 4. Resolution of a test mixture of probable 1,2-DMH metabolites on μBondapak C_{18}. Two μBondapak columns, each 4 mm × 30 cm, connected in series, were eluted isocratically with 1% ethanol at a rate of 1 ml/min. Absorbancy of the effluent was monitored at 205 nm. Inserts show the elution volumes of [^{14}C]-formaldehyde and [^{14}C]-methanol which were run separately. Pressure, 2000 p.s.i.

ample, for AOM, the number of theoretical plates was found to be 330 per m on C_{18}/Corasil and approximately 12,000 per m on μBondapak C_{18}. The high efficiency of μBondapak C_{18} permitted separations such as those of formaldehyde and methanol (Fig. 4), not possible on C_{18}/Corasil.

For some purposes, the characteristic strong retention of 1,2-DMH by C_{18}/Corasil and μBondapak C_{18} proved to be a disadvantage. In particular with samples containing [^{14}C]-1,2-DMH considerable trailing of [^{14}C]-1,2-DMH resulted (Fig. 3) with both 0.05 *M* acetic acid and 1% ammonium carbonate elution. The extensive elution of the columns necessary for the complete recovery of [^{14}C]-1,2-DMH increased the time required for a separation. Moreover, a further washing of the column with the initial solvent, 1% ethanol, was necessary in order to remove the acetic acid or ammonium carbonate prior to the application of the next sample.

In a search for a column packing which would not exhibit these undesirable characteristics, we observed that AOM and MAMOAc were adsorbed, in aqueous solution, to polystyrene type resins (*e.g.*, Amberlite XAD-2; Rohm & Haas, Philadelphia, Pa., U.S.A.). This suggested that a polystyrene anion-exchange resin would serve well in this application since, at pH below neutrality, protonated 1,2-DMH would elute early due to ion exclusion while the azo and azoxy compounds would be retained. This expectation was borne out; a separation of 1,2-DMH, formaldehyde, MAM, AOM, AM and MAMOAc on a column of Aminex A-27 (acetate form) eluted with 0.01 *M* sodium acetate (pH 5.6) is shown in Fig. 5. While methanol was not separated from formaldehyde, adequate separation of the azo and azoxy compounds with a full recovery of [^{14}C]-1,2-DMH was obtained. For reproducible elution volumes, especially of the late eluting MAMOAc, close temperature control of the Aminex A-27 column was found to be necessary. This was achieved by circulating water at 29.5° in a glass jacket surrounding the column.

An application of the Aminex A-27 column to the detection and separation of urinary metabolites derived from [^{14}C]-1,2-DMH is shown in Fig. 6. It should be

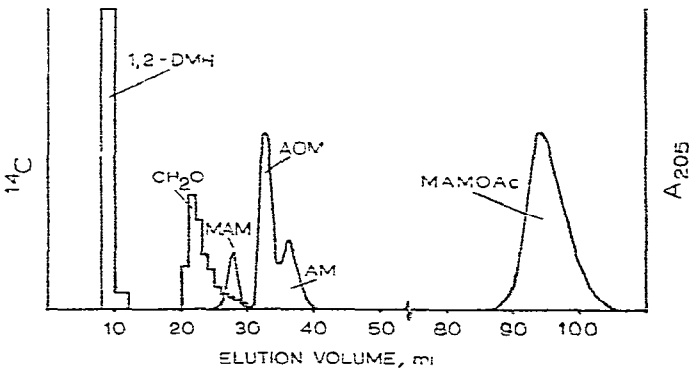


Fig. 5. Separation of a test mixture of [^{14}C]-1,2-DMH and probable metabolites on Aminex A-27. A 3/8 × 24 in. column of Aminex A-27 (Ac^- form) was eluted at a rate of 1 ml/min with 0.01 *M* sodium acetate (pH 5.6). Azo and azoxy compounds were detected by monitoring absorbancy at 205 nm. [^{14}C]-1,2-DMH and [^{14}C]-formaldehyde were detected by liquid scintillometry in collected 1-ml or 0.5-ml fractions. The column was run at a constant temperature of 29.5°. Elution rates of up to 3 ml/min could be used without decreasing separation efficiency. Pressure, 300 p.s.i. at 1 ml/min.

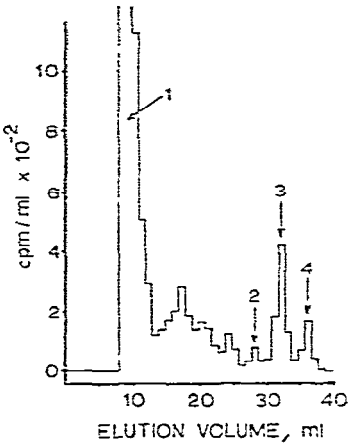


Fig. 6. Elution profile of [^{14}C]-labeled urinary metabolites derived from [^{14}C]-1,2-DMH. A 100-μl urine sample freshly collected from a male rat injected 2 h previously with 10 μCi of [^{14}C]-1,2-DMH (subcutaneous; total dose 200 mg per kg body weight) was applied to an Aminex A-27 column and eluted with 0.01 *M* sodium acetate (pH 5.6) as in Fig. 5. 1-ml fractions were collected and radioactivity determined by liquid scintillometry. 1 = 1,2-DMH; 2 = MAM; 3 = AOM; 4 = AM.

noted that up to about six consecutive 100-μl samples of urine could be run without noticeable degradation of performance of this column. After six runs, the column was regenerated overnight using 4 *M* sodium acetate (pH 5.6) usually at a low flow-rate (0.1–0.2 ml/min).

The HPLC methods described are currently being used for the analysis of physiological fluids and tissue extracts of animals treated with 1,2-DMH as well as to assay for enzymes catalyzing the activation and detoxification reactions of this powerful carcinogen.

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