Journal of Chromatography, 177 (1979) 118–121
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CHROM. 12,070

Note

Methylation of 6-mercaptopurine using trimethylanilinium hydroxide

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(Received May 18th, 1979)

The quantitative determination of 6-mercaptopurine (6-MP) in serum using gas chromatography (GC)–chemical ionization mass fragmentography was investigated by Pantarotto *et al.*[1]. To prepare a volatile derivative of 6-MP for analysis, the "flash methylation" reaction with trimethylanilinium hydroxide (TMAH) was employed[2]. The authors reported that only one GC peak was observed for this reaction and the structure assigned to the methylated product was 9-methyl-6-methylmercaptopurine (9-M-6-MMP)[1]. However, in attempting to utilize this assay procedure for 6-MP, we obtained three major GC peaks from the "flash methylation" procedure. The three compounds corresponding to the GC peaks were shown to be dimethylated derivatives of 6-MP by combined gas chromatography–mass spectrometry (GC–MS). Using the fragmentation patterns of the various dimethylated derivatives of 6-MP which had been previously reported[3], we tentatively assigned the structures of 3-M-6-MMP, 7-M-6-MMP and 9-M-MMP to the methylated compounds. These three compounds were then synthesized according to the literature[4–6]. Confirmation of the assigned structures was accomplished by comparing the mass spectrum of the prepared standards with those of the compounds obtained from the methylation reaction.

EXPERIMENTAL

Materials and methods

6-MP, 6-methylmercaptopurine and 6-chloropurine were obtained from Sigma (St. Louis, Mo., U.S.A.); dimethyl sulfate, 1-heptanesulfonic acid sodium salt, and methyl *p*-toluenesulfonate from Eastman-Kodak (Rochester, N.Y., U.S.A.); sodium sulfhydrate and N,N-diethylacetamide from Fisher Scientific (Fair Lawn, N.Y., U.S.A.); methyl iodide and Diazald from Aldrich (Milwaukee, Wisc., U.S.A.); and Methelute (0.2 *M* TMAH in methanol) from Pierce (Rockford Ill., U.S.A.). Solvents employed were reagent grade and were used without further purification.

Instrumentation

A Varian gas chromatograph (Model A-90-P) equipped with an empty stainless-steel column (122 cm × 3.2 mm I.D.) was used for collecting the reaction product from the flash methylation reaction of 6-MP with TMAH. The injector

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temperature was maintained at 300° and the flow-rate of the carrier gas (helium) was 40 ml/min. A Waters Assoc. (Milford, Mass., U.S.A.) ALC/GPC 244 high-performance liquid chromatograph equipped with a U6K injector was used for the separation and isolation of the products from the flask methylation reaction. The column was a 300 × 4 mm prepacked stainless-steel column containing octadecyltrichlorosilane (μBondapak C_{18}; Waters Assoc.). The degassed mobile phase was a paired-ion solution containing 0.005 *M* 1-heptanesulfonic acid sodium salt, 1% glacial acetic acid and 30% methanol in distilled water. The temperature was ambient and the solvent flow-rate was 1.0 ml/min. A wavelength kit for monitoring at 313 nm was used. The detector was set at a sensitivity of 1.0 a.u.f.s. and the chart speed of the recorder was 30.5 cm/h. The GC–MS system used in this study was a Pye Unicam Model 104 gas chromatograph coupled to an AEI MS-30 single beam mass spectrometer. The operating conditions of the mass spectrometer were: electron impact mode, source temperature 180°, source pressure $3 \cdot 10^{-7}$ Torr, electron current 100 μA, electron energy 70 eV and scan speed 10 sec/decade. The gas chromatograph was fitted with a 275 cm × 2 mm I.D. glass column packed with 3% OV-17 on 100–120 mesh Gas-Chrom Q and used after silylation with Silyl-8 (Pierce). The port temperature was maintained at 300° and all column temperature programs were isothermal at 250° with the carrier gas (helium) at a flow-rate of 15 ml/min.

Procedure

A 1.0-ml sample of 6-MP in methanol (1 mg/ml) was placed in a small screw-capped vial and the solvent was removed by a gentle stream of nitrogen. After the addition of 1.0 ml of TMAH to the dry residue, the vial was capped and the contents were mixed thoroughly. The mixture was then slowly injected into the Varian gas chromatograph in 50-μl portions. The products of this flash methylation reaction were collected by trapping the volatile components at the exit port using a tube cooled with a dry ice–propylene glycol mixture. When the collected products were analysed using the high-performance liquid chromatography (HPLC) instrument, there were found to be three major peaks in the chromatogram (Fig. 1). Using HPLC (50-μl sample loads), the components of the reaction mixture were separated and then isolated by collecting the effluent fractions corresponding to each peak. The HPLC solvent was then removed from each fraction using a gentle stream of nitrogen. Methanol (200 μl) was added to the dry residue and the contents mixed thoroughly. After centrifuging for a few minutes, the clear supernatant was poured off into a clean test tube. The sample was then injected into the GC–MS system to obtain its spectrum. The prepared standards (3-M-6-MMP, 7-M-6-MMP and 9-M-6-MMP) were separately dissolved in methanol (1 mg/ml) and their mass spectra were obtained as above.

RESULTS AND DISCUSSION

The alkylation chemistry of 6-MP has been well documented in the literature[7–10]. Using other methods, it has been shown that the N^1, N^3, N^7 and N^9 positions are all susceptible to methylation, depending on the reaction conditions. The use of TMAH as a methylating reagent for the quantitative measurement of 6-MP in serum has also been reported by Bailey *et al.*[11]. These authors tried many different reagents

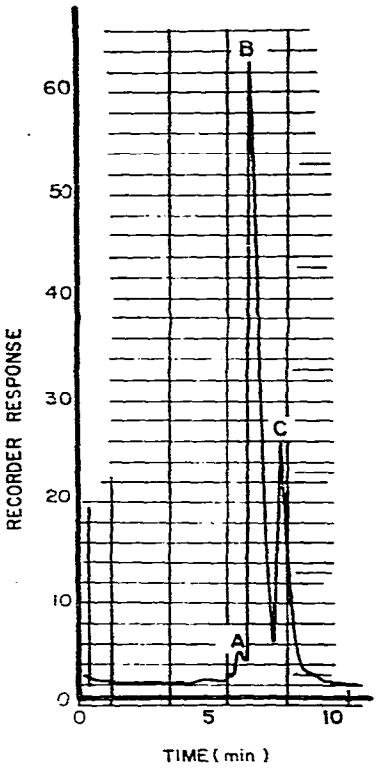

Fig. 1. HPLC chromatogram of products obtained from the reaction of TMAH with 6-MP.

for preparing volatile derivatives of 6-MP and came to the conclusion that the only agent capable of producing a suitable derivative was TMAH. For their assay procedure, the derivatization was done at 135° for 30 min prior to GC injection instead of flash methylation, which is the recommended method in using the reagent[12]. However, a detailed analysis of the reaction mixture using TMAH by this method was not done and the authors reported only one GC peak[11]. The results of the present study indicate that the flash methylation of 6-MP using TMAH does not produce a single compound as previously reported[1] but instead a mixture of isomeric products. When the analytical method utilizes a derivatization procedure, it can only be useful if the reaction gives one product or a mixture of products in a constant ratio. To test the variability of the products formed during the flash methylation reaction, different concentrations of 6-MP (1000, 100 and 10 μg/ml) in TMAH were prepared. The three methylated products were obtained in the ratio of approximately 67:28:5 (9-M-6-MMP:7-M-6-MMP:3-M-6-MMP) over the entire range. This corresponds closely to the results of Rosenfeld *et al.*[10] who found a 70:30 ratio (9-M-6-MMP:7-M-6-MMP) in the alkylation of 6-MP with methyl iodide in methylene chloride. Although the use of TMAH has been found not to be selective in the formation of only one volatile derivative of 6-MP, the analytical method still has validity since the ratio of the products in the methylation reaction was found to be relatively constant.

ACKNOWLEDGEMENTS

This investigation was supported in part by Research Grant RR-08111 from the National Institutes of Health, Minority Biomedical Support Program and in part by the National Cancer Institute, Bethesda, Md., U.S.A.

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