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Note

Approach to the quantitative analysis of nucleotides by gas chromatography—mass spectrometry

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Nucleotides are not easily amenable to gas chromatographic analysis [1] and for this reason the specificity and sensitivity of gas chromatography—mass spectrometry (GC—MS) with selected ion monitoring (SIM) [2] is rarely employable for nucleotide analysis. High-performance liquid chromatography (HPLC) has instead recently become a powerful and convenient method for the analysis of nucleotides [3]. The specificity and sensitivity of HPLC analysis of nucleotides may, however, be ultimately limited since UV detection is commonly employed [4]. In an effort to make nucleotides more amenable to GC—MS analysis with SIM, we have devised a general scheme for the analysis of nucleosides and nucleotides (so far, ribonucleoside monophosphates) from biological media.

The analytical scheme is evaluated with the analysis of nucleotide anabolites of the antineoplastic [5] and immunosuppressive [6] agent 6-mercaptopurine (6-MP). This drug must be taken up by cells and converted by hypoxanthine—guanine phosphoribosyl transferase (HGPRT) directly into 6-mercaptopurine riboside-5′-phosphate (MPRP), a potent inhibitor of nucleic acid synthesis. MPRP can then be methylated in vivo at the sulfur to give 6-methylmercaptopurine riboside-5′-phosphate (MMPRP), another active anabolite. These ribonucleoside monophosphates may be dephosphorylated in vivo to give the corresponding ribosides, 6-mercaptopurine riboside (MPR) and 6-methylmercaptopurine riboside (MMPR). This enzymatic dephosphorylation may be important in the development of clinical resistance to 6-MP [7]. It is important,

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therefore, that the analytical methodology devised should be capable of measuring both the nucleosides of 6-MP as well as the active nucleotides.

MATERIALS AND METHODS

Reagents

MPR, MMPR, other nucleosides, MPRP, acid phosphatase and 5′-nucleotidase were obtained from Sigma (St. Louis, MO, U.S.A.). Sodium hydride, sodium methoxide, 2-mercaptoethanol, dimethylsulfoxide (DMSO) and methyl iodide were purchased from Aldrich (Milwaukee, WI, U.S.A.). Perchloric acid was obtained from Pfaltz and Bauer (Stamford, CT, U.S.A.) and solvents were obtained from Baker (Phillipsburg, NJ, U.S.A.). Amberlite XAD-4 resin was purchased from Supelco (Bellefonte, PA, U.S.A.). Dideuterated nucleoside and nucleotide analogs were prepared as previously described [8].

Extraction from blood

Nucleosides and nucleotides were extracted from blood essentially by the method of Nelson et al. [9], which is itself a modification of Caldwell's procedure [10]. That is, one drop of 2-mercaptoethanol was added to 2.5 ml of blood to prevent oxidation of mercaptopurines. Five ml of 0.5 *M* perchloric acid solution were added to the 2.5 ml of whole blood and mixed thoroughly. The brown precipitate formed was removed by centrifugation and re-extracted with another 5 ml of 0.5 *M* perchloric acid. The perchloric acid extracts were combined and potassium hydroxide solution was added until the solution attained pH 6—6.5. The resulting precipitated potassium perchlorate was removed by centrifugation and washed with 5 ml of distilled water. The combined aqueous extracts were reduced in volume to ca. 1 ml.

Methanol-washed XAD-4 resin (40—80 mesh) was poured into a Pasteur pipette which contained a glass wool plug and had an attached reservoir. The column was then flushed with 20 ml of methanol followed by 40 ml of water. The 1-ml aqueous blood extract was added to the top of the XAD-4 column and the column was washed with 10 ml of distilled water to elute the nucleotide fraction. The column was then washed with 8 ml of a 20% aqueous ethanol solution. This eluted the nucleoside fraction which was then dried. To the eluted aqueous nucleotide fraction were added about 3 units of acid phosphatase and the solution was incubated at 37°C for 15 min. The solution was then poured onto another XAD-4 Pasteur pipette column. The column was washed with 10 ml of distilled water. The nucleosides which had been enzymatically produced from the nucleotides were then removed from the column with 8 ml of 20% aqueous ethanol and the eluate was dried.

Methylation of nucleosides

Nucleosides were either methylated by the methylsulfinyl carbanion/methyl iodide method [11] or by the use of sodium methoxide and methyl iodide. That is, for the latter method, the nucleosides were dissolved in 500 μl of DMSO in a 4-ml sample vial, and to this solution was added ca. 0.5 mg of sodium methoxide powder. After mixing well with shaking, 50 μl of methyl iodide were added. The vial was tightly closed with a PTFE-lined cap and

heated with shaking in a steam bath for 5 min. The reaction was then stopped by the careful addition of 500 μl of distilled water. The derivatized nucleosides were then extracted with 500 μl of chloroform which was subsequently washed with water to remove DMSO and dried with granular anhydrous sodium sulfate. If desired, the final volume of chloroform was reduced with a nitrogen stream before GC—MS analysis. This methylation procedure is essentially that which Bryant and Klein [12] used for the N-methylation of purines and pyrimidines.

GC—MS analysis

The permethylated nucleosides were analyzed on a Dupont 21-492 double-focusing mass spectrometer, equipped with a chemical ionization source and a four-channel selected ion monitoring system, and interfaced to a Varian 2740 gas chromatograph via an all-glass system with a jet separator. Methylated nucleosides were admitted to the mass spectrometer for selected ion monitoring via a 6-ft glass column packed with 3% OV-101 or 3% OV-17 on Supelcoport. Operating temperatures were: injection port, 280°C; column, 260°C; GC—MS interface and jet separator, 280°C; MS source, 220°C.

Thin-layer chromatography

Thin-layer chromatography (TLC) was performed on Baker-flex silica gel IB2-F precoated sheets with 0.5 *N* ammonium sulfate—95% ethanol (1:1) as eluent. R_F values for MPRP and MPR were 0.68 and 0.75, respectively.

RESULTS AND DISCUSSION

The overall extraction and analysis scheme for nucleosides and nucleotides from tissue such as blood is outlined in Fig. 1. Nucleotides and nucleosides are rapidly separated from each other by XAD-4 resin [13] as illustrated in Fig. 2, which shows the elution of a spiked blood extract containing MPRP, MPR and MMPR. MPRP was eluted with ca. 10 ml of distilled water followed by elution of MPR with ca. 8 ml of 20% ethanol—water and finally elution of MMPR with 50% ethanol—water. The elution profiles were followed by passing the eluate through a quartz flow-cell placed in a Coleman 101 variable-wavelength UV spectrophotometer. MPRP and MPR were monitored at 321 nm while MMPR was monitored at 293 nm. By using 50% aqueous ethanol instead of 20%, the two ribonucleosides MPR and MMPR can be eluted together if desired.

The nucleoside monophosphates eluted from the XAD-4 column were cleaved to their ribonucleosides by incubation with acid phosphatase which was added directly to the eluate. Cleavage is complete after 15 min at 37°C, as determined by TLC. Although pH 4 is optimum for acid phosphatase activity and the eluate can be adjusted to this pH if desired, the enzyme is still active enough at more neutral pH to effectively cleave all the nucleoside monophosphate. Therefore, since elution of nucleotides was by distilled water, pH adjustment was not necessary. Note that buffers are also avoided. There is the distinct possibility that acid phosphatase may not be specific for monophosphates and can cleave higher phosphates to the riboside which would inflate the measurement of the amount of monophosphate present. Hence, instead of using acid phosphatase we have used 5′-nucleotidase, which is a much more

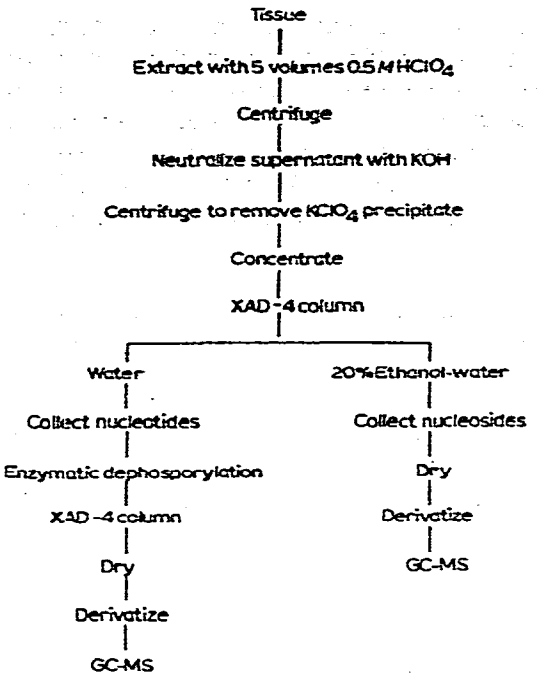

Fig. 1. Extraction, separation, derivatization and analysis scheme for tissue nucleosides and nucleotides.

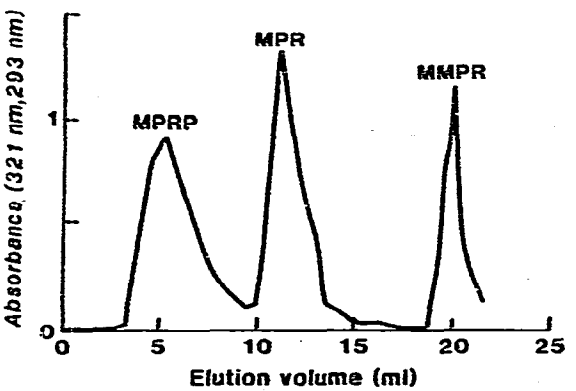

Fig. 2. Separation of mercaptopurine nucleosides and nucleotides on a 0.5 cm × 4.0 cm bed of Amberlite XAD-4 resin. Mercaptopurine riboside-5'-monophosphate (MPRP), mercaptopurine riboside (MPR), and methylmercaptopurine riboside (MMPR) were added to whole blood and extracted with perchloric acid. The neutralized, concentrated extract (1.5 ml) was applied to the column and eluted with 10 ml of distilled water, followed by 8 ml of 20% ethanol—water and finally with 50% ethanol—water. MPRP and MPR were monitored at 321 nm while MMPR was monitored at 293 nm.

specific enzyme. pH 9 is optimal for this enzyme but again it is active enough to easily cleave all the 5'-monophosphate to nucleoside at neutral pH and at 37°C for 15 min.

After enzymatic dephosphorylation, the resulting nucleoside was very effectively cleaned up for permethylation derivatization by passing the solution through another small XAD-4 column. After adding the solution to the column, it was first washed with distilled water which removed water-soluble material from the column before the nucleosides were removed with 20% aqueous ethanol.

Permethylation can be accomplished by the methylsulfinyl carbanion/methyl iodide procedure [11]. This is an efficient reaction for most nucleosides. However, the overall derivatization procedure is quite time-consuming since a total of 2 h is required for the overall reaction before extraction of the product. Further, fresh reagent solution must be prepared at intervals. We therefore investigated the possibility of using a simpler methylation procedure. Bryant and Klein [12] used sodium methoxide and methyl iodide in DMSO to N-methylate purine and pyrimidine bases in a convenient and rapid procedure. We have found that the same procedure is effective for the permethylation of nucleosides. The methylation yield was not quite as consistently good as with the methylsulfinyl carbanion method as illustrated in Fig. 3. The gas chromatograms are of an equimolar mixture (ca. 1 $\mu g/\mu l$) of uridine, deoxyadenosine, adenosine and MPR derivatized by the two permethylation procedures. The overall yield of these reactions, including extraction, for MPR relative to a standard solution of permethylated MPR was approximately 80% for the methylsulfinyl carbanion method and about 65% for the sodium methoxide method. However, the convenience and speed of the latter method may outweigh the slightly lower derivatization yield. The sodium methoxide method generally worked as well as the methylsulfinyl carbanion method with adenosine, deoxyadenosine, thymidine, uridine, cytidine and deoxycytidine, MPR, and 5-fluorodeoxyuridine (5-FU), but not with guanosine or deoxyguanosine.

Permethylated nucleosides were analyzed by GC—MS using chemical ioniza-

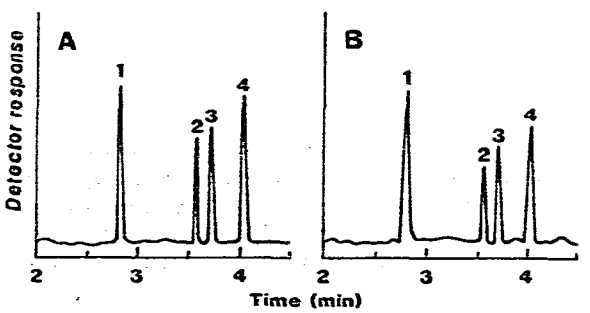

Fig. 3. Gas chromatograms of a mixture of uridine (1), deoxyadenosine (2), adenosine (3), and mercaptopurine riboside (4), derivatized by the methylsulfinyl carbanion/methyl iodide method (A) and the sodium methoxide—methyl iodide method (B), respectively. The traces are recordings of the total mass spectrometric ion current obtained when the derivatized mixtures were injected on a 6-ft. glass column packed with OV-101 and operated at 180—300°C at 20°/min.

tion and selected ion monitoring of the protonated molecular ions [14]. Although isobutane was routinely used for chemical ionization it is worth noting that the response of the protonated molecular ion was decreased to 63% when methane was used as the reagent gas. When ammonia was used the response increased to 143% relative to isobutane. Therefore, ammonia appears to be the most suitable reagent gas for this type of analysis.

Fig. 4 is an illustration of the effectiveness of the overall extraction, derivatization and analysis procedure. 12.5 nmoles (4.8 μg) of MPRP-5',5'-d_2 were added to 5 ml of whole blood along with 5 nmoles (1.8 μg) of MPRP. This represented a concentration of MPRP of 1 nmole/ml or 1 μM. After extraction and derivatization, which was carried out on two separate 2.5-ml blood samples, 2 μl were taken from a final combined volume of 20 μl of chloroform and injected on the GC—MS system to produce the SIM trace of Fig. 4C. Traces for standard permethylated MPR-5',5'-d_2 (Fig. 4B) and an extracted and derivatized blood blank (Fig. 4A) are also illustrated. It should be noted that the response for 1 nmole/ml from 5 ml of whole blood was obtained with only 10% of the final solution, without the instrument being set at its limit of sensitivity (noiseless baseline), and using the slightly less sensitive reagent gas isobutane. Although this was an extraction from a spiked blood sample and may not reflect the real situation, it is apparent that the nM range is within easy reach.

We are currently pursuing a number of modifications of the procedure discussed to increase the overall sensitivity and to minimize the time of analysis.

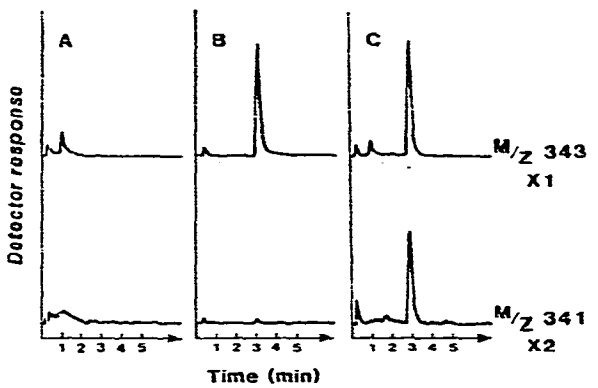

Fig. 4. GC—MS SIM traces of m/z 341 and 343. (A) Extracted blood blank; (B) dideuterated mercaptopurine riboside monophosphate (MPRP-5',5'-d_2) enzymatically dephosphorylated and permethylated by the sodium methoxide—methyl iodide procedure; (C) 1 nmole/ml of MPRP with 2.5 nmoles/ml of MPRP-5',5'-d_2 added to 5 ml of whole blood and extracted, chromatographed, derivatized (sodium methoxide—methyl iodide method), and analyzed as described in the text.

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