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Note

Determination of pentamethylmelamine and hexamethylmelamine in plasma and urine by nitrogen–phosphorus gas–liquid chromatography

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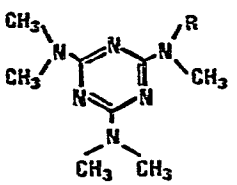
Pentamethylmelamine (PMM, Fig. 1) is a water-soluble analog of the antitumor agent, hexamethylmelamine (HMM, Fig. 1). HMM has been studied in clinical trials[1] based on activity against several tumor test systems including Crocker mouse sarcoma 180[2] and Walker rat carcinosarcoma 256[3]. It is active against human small cell lung carcinoma, ovarian adenocarcinoma, lymphoma and breast cancer[1,4].



R=H PMM
R=CH_3 HMM

Fig. 1. Structures of pentamethylmelamine and hexamethylamine.

Because of limited water solubility, HMM is administered orally. Gastrointestinal toxicity (especially nausea and vomiting) has limited its clinical use[4–6]. Metabolism studies have shown that HMM is converted primarily to demethylated metabolites and that PMM is the first intermediate in this pathway[6–9]. PMM has antitumor activity similar to HMM against a wide variety of animal tumor models and was selected by the National Cancer Institute as the most promising HMM analog with increased water solubility[6]. While clinical interest in HMM continues, PMM is being studied in clinical trials by intravenous administration in hopes of achieving significant activity with reduced gastrointestinal toxicity.

Thin-layer chromatographic[9–11] methods used for identification of HMM and metabolites do not provide the sensitivity required for disposition studies. A quantitative method for HMM and metabolites based on isolation and separation of the compounds with ion-exchange chromatography and ultraviolet absorption detection[7,8,12] was also not sufficiently sensitive for our studies. This technique had been

applied previously to the determination of HMM in human plasma following oral administration of HMM (4–12 mg/kg), and parent drug was observed only at the first sampling time at the highest dose[9]. While gas chromatographic (GC)–mass spectrometric methods have been used for analysis of HMM and metabolites[7,8], we did not wish to employ this approach for routine sample analysis. Several groups have described GC procedures for the analysis of HMM and metabolites[7,11] but did not apply them to quantitative assays.

Because methods described in the literature for quantitative determination of HMM and related compounds such as PMM did not appear adequate for our purposes, we have developed a GC method for PMM and HMM in biological fluids. Using a sensitive and specific nitrogen detector, we can quantitate less than 200 ng/ml HMM or 25 ng/ml PMM in plasma or urine. Quantitative analysis of PMM is accomplished using HMM as an internal standard, that of HMM with standard curves constructed by addition of HMM to control samples. We have applied the PMM assay to preliminary disposition and metabolism studies of this agent in patients following intravenous administration of this agent.

MATERIALS AND METHODS

All glass-distilled solvents were obtained from Burdick & Jackson Labs. (Muskegon, Mich., U.S.A.). Pentamethylmelamine hydrochloride and hexamethylmelamine hydrochloride were provided by the Drug Synthesis and Chemistry Branch, Division of Cancer Treatment, National Cancer Institute (Bethesda, Md., U.S.A.). GC analyses were carried out on a microprocessor-controlled Hewlett-Packard 5480A gas chromatograph equipped with a nitrogen–phosphorus detector (NPD). Silanized glass columns (2 ft. × 2 mm I.D.) were packed with 10% Carbowax 20M–2% KOH on 80–100 mesh Chromosorb W AW (Supelco, Bellefonte, Pa., U.S.A.) and conditioned overnight (215°) prior to analysis. Injector and detector temperatures were 235 and 300°, respectively. Hydrogen gas flow-rate was 3 ml/min, air 90 ml/min and nitrogen 30 ml/min (Air Products, Allentown, Pa., U.S.A.). An oven temperature program (170–185°, 10°/min) was used for three successive analyses, followed by 15–20 min at 190° to elute contaminants prior to injection of the next three samples.

Plasma and urine analysis of PMM

A 1-ml volume of heparinized plasma or urine (stored at −20° until analysis) was added to a glass* 16 × 125 mm screw-capped centrifuge tube and HMM (0.5 or 2.0 μg in 10 μl methanol) added as internal standard. The pH was adjusted to 11 with 1.0 *N* sodium hydroxide, 6 ml toluene added, and the samples shaken for 20 min on a mechanical shaker. Following centrifugation (1000 *g*, 15 min) the toluene layer was transferred to a 12-ml conical centrifuge tube and the solvent removed under a stream of nitrogen. The samples were reconstituted in appropriate volumes of toluene (usually 10–50 μl) and 1-μl aliquots injected for GC analysis. The concentration of PMM was determined by peak area ratios of drug to internal standard compared to values obtained from plasma or urine standard curves.

* All glassware was silanized prior to use.

Plasma and urine analysis of HMM

Analysis of HMM was carried out as described for PMM but without the addition of an internal standard. Concentrations of HMM in samples were determined by comparison of peak areas to those of a standard curve obtained by addition of known amounts of HMM to control plasma and urine samples.

RESULTS AND DISCUSSION

Based on previous results with HMM[7,11] and studies with triazine herbicides[13], we studied the GC properties of PMM and HMM with a number of stationary phases. Tailing, a common observation with underivatized amines[14], and poor separation of the structurally similar melamines, PMM and HMM, were the major problems. Analysis with a 6-ft. 10% Apiezon L–2% KOH column (Supelco) resulted in sharp, symmetrical peaks, but would not satisfactorily resolve PMM and HMM. Similarly, a 6-ft. methyl silicone phase deactivated for amines (3% SP-2100 DB, Supelco) provided inadequate separations. A 6-ft. 10% Carbowax 20M–2% KOH gave excellent separation, but unacceptably long retention times and significant broadening of the peaks. A 2-ft. column with this phase, however, provided excellent chromatographic properties for PMM and HMM. The NPD permitted quantitation of as little as 500 pg of PMM or HMM injected on column.

Toluene provided optimal extraction of PMM and HMM from aqueous solutions, with recoveries of 60–80%. Both PMM and HMM are somewhat volatile, and care must be taken in sample concentration not to allow dry samples to remain under the nitrogen stream for prolonged periods of time. Chromatograms of a control patient plasma sample and one containing 800 ng/ml PMM (with 2 μg HMM as internal standard) are shown in Fig. 2A and B, respectively. Chromatograms of urine

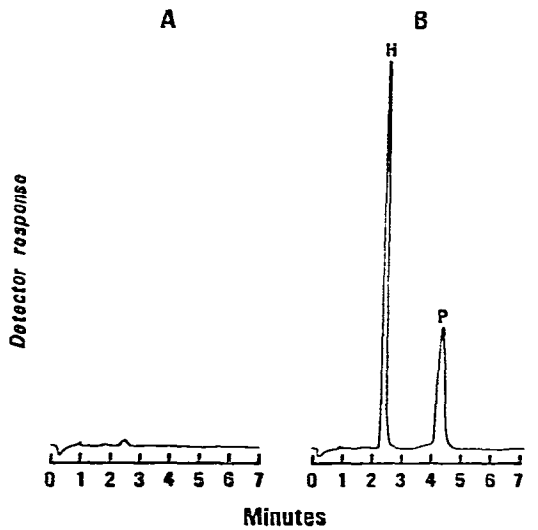


Fig. 2. Chromatograms of (A) control patient plasma sample and (B) patient plasma sample to which 2 μg HMM was added as internal standard and found to contain 800 ng/ml PMM. Samples were analyzed as described in Materials and methods.

samples are similar (not shown). The control plasma sample (Fig. 2A) contained a small amount of HMM. We found that both compounds were carried over both by glassware and syringes. Thorough rinsing and dishwashing prevents this problem. Use of the NPD and toluene extraction provide excellent specificity, with only one contaminant in both plasma and urine appearing as a broad peak at 23 min. Temperatures above 190° cannot be used to elute the contaminant because of limited stability of the stationary phase (conditioned at 215°). Maintenance of the column temperature at 190° for 15–20 min after three successive sample analyses eliminated contaminant buildup and maintained column performance.

Plasma and urine standard curves for PMM with 2 μg HMM internal standard are shown in Fig. 3. The curves are linear ($r^2 > 0.99$) and reproducible. We can quantitate 25 ng/ml using 500 ng HMM as internal standard. We prepared standard curves for HMM in plasma and urine by adding HMM to control samples. These curves were linear ($r^2 > 0.99$), but the sensitivity was limited (200 ng) by variation of replicates at low concentrations. In addition, we found it necessary to prepare a standard curve daily for HMM analyses due to variability in the response of the NPD.

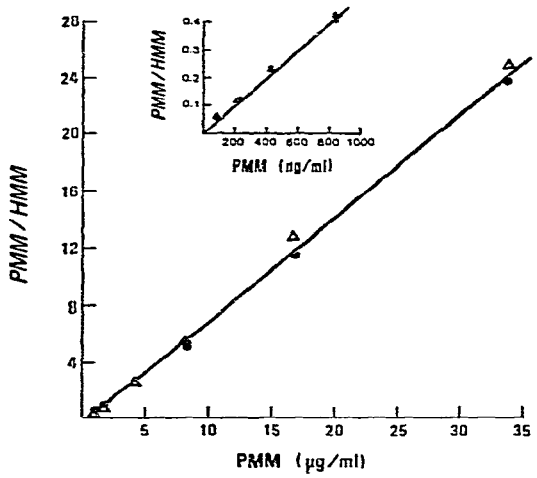


Fig. 3. Standard curves for PMM in plasma (●) and urine (△) with 2 μg HMM as internal standard.

The PMM assay was applied to preliminary disposition and metabolism studies in two patients who received 80 mg/m^2 PMM administered by intravenous infusion over a 2-h period. This prolonged infusion was used to avoid acute central nervous system toxicity associated with rapid infusion in large animals[6]. The plasma elimination curves for these patients are shown in Fig. 4. The sensitivity of the assay was crucial for analysis since peak plasma levels were about 1 μg/ml. Urinary recovery of PMM was low, less than 1% in 24 h. The plasma and urine of these patients contained several metabolites. They have not been identified, but are most likely demethylated PMM metabolites. A number of demethylated analogs of HMM (including PMM) have been identified in the urine of animals and man following administration of HMM[7–9,12].

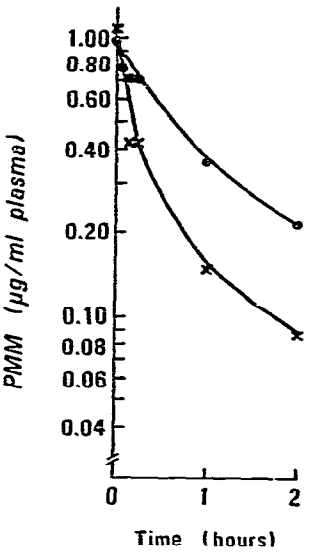


Fig. 4. Plasma time–concentration curves of PMM in two patients following a 2-h intravenous infusion of PMM (80 mg/m²).

The mechanism of action of PMM and HMM is not known at this time. Neither compound reacts with nitrobenzylpyridine[7] suggesting these compounds do not act as alkylating agents. It has, however, been suggested that a metabolic intermediate of HMM (and by analogy PMM) metabolism may be an alkylating agent[7], and binding of both ring- and methyl-labeled HMM to liver has been demonstrated following administration of HMM to mice[9].

Our studies have shown that with a sensitive and specific assay, one can detect significant quantities of parent drug in plasma following administration of PMM to humans. In addition, PMM is extensively matabolized, probably by the liver, and these metabolites can be detected in plasma and urine. We are continuing our pharmacokinetic and metabolic studies of PMM in man and animals.

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