**N-Demethylation of the Antineoplastic Agent Hexamethylmelamine by Rats and Man**

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**SUMMARY**

The metabolism and physiological distribution of hexamethylmelamine (HMM), an effective human anticancer chemotherapeutic agent, was studied in cancer patients and in rats. Administration of HMM-methyl-14C to two patients p.o. was followed by the prompt appearance of respiratory 14CO2 within 1 hr, accumulating to 9% of administered dose in 6 hr. After 72 hr, 29% of the radioactivity was recovered in the urine, and 0.5% was recovered in the feces. In rats, p.o. and i.p. administration of HMM-14C led to the recovery of 13 and 30%, respectively, of the dose as 14CO2 in 24 hr. After 72 hr, 58% of the p.o. dose of radioactivity was recovered, 16% as 14CO2, 38% in the urine, and 4% in the feces; while in the same interval, 80% of the i.p. dose was recovered, 33% as 14CO2, 42% in the urine, and 5% in the feces. No unmetabolized HMM was detected in urine; instead four distinct compounds could be chromatographically separated. The major urinary metabolites in patients and rats exhibited identical chromatographic and spectroscopic properties. Use of gas chromatography and mass spectrometry led to the identification of the major urinary metabolites as N2,N4,N6-trimethylmelamine, N2,N4-dimethylmelamine, monomethylmelamine, and melamine. Thus, N-demethylation appears to be of major significance in the metabolism of HMM in man and in rats. HMM and its metabolites did not demonstrate significant alkylating activity, as evidenced by their failure to react with 4-(p-nitrobenzyl)pyridine.

**INTRODUCTION**

HMM has exhibited slight but definite tumor-inhibiting properties against Crocker mouse Sarcoma 180 (12) and Walker rat Carcinoma 256 (20). However, these animal screening tests showed that the tumor-inhibiting properties of HMM were inferior to those of 2 structurally similar s-triazine compounds, TEM and trimethylolmelamine [2,4,6-tris(methylolamino)-s-triazine] (12, 20, 21).

Clinical trials with HMM were initiated by Wilson and de la Garza (36) and by Louis et al. (24). Phase II studies with HMM were conducted, and the response rate of patients demonstrated HMM to be worthy of further clinical study especially for lung carcinoma. The clinical literature for HMM has been recently reviewed by Blum et al. (8).

Although HMM has been used in several clinical trials with cancer patients, information concerning the mechanism of action of this drug and its metabolism is sparse. Studies in our laboratory with HMM have included the development of an ion-exchange method for the isolation and quantitation of the plasma and urinary levels of HMM (10) and an investigation of the metabolism of HMM in man and rats. This paper describes the metabolism of HMM-methyl-14C and reports the identification of the human and rat urinary metabolites of HMM.

**MATERIALS AND METHODS**

**Chemicals.** Unlabeled HMM was provided by the Clinical Branch, Collaborative Research, National Cancer Institute, USPHS. Samples of the other methylmelamine compounds were generously provided by Dr. A. B. Borkovec of the Entomology Research Division of the United States Department of Agriculture, Beltsville, Md. Melamine (Aldrich Chemicals, Milwaukee, Wis.) was recrystallized from water. TEM was synthesized (38) from cyanuric chloride (2,4,6-trichloro-s-triazine, Eastman Organic, Rochester, N. Y.) and ethylenimine (K and K Laboratories, Plainview, N. Y.).

The synthesis of HMM-methyl-14C was based on a method used by Kaiser et al. (22). To a flask containing 3 mmole (554 mg) of cyanuric chloride and 75 ml of acetone were added 8.7 mmole (710 mg) of dimethylamine hydrochloride (Aldrich Chemicals) and 0.3 mmole (24 mg, 0.5 mCi) of dimethylamine-14C hydrochloride (New England Nuclear, Boston, Mass.). After the addition of 8 ml of 2 N NaOH solution, the reaction mixture was refluxed for 3 hr, and the acetone was then removed by distillation. The remaining aqueous solution was allowed to cool, and the HMM was extracted with chloroform. After washing with water, the chloroform was evaporated, and the HMM was recrystallized twice from methanol, yielding 260 mg (41%) of crystalline HMM-methyl-14C, m.p. 170–172°.
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Phase III clinical cancer chemotherapy trials with HMM. Two patients were given HMM-methyl-\(^{14}\)C, dissolved in a small portion of 6 \(\mu\) HCl and added to orange juice (pH 2.3), p.o. in the morning. Patient 1 received 110 mg (98.6 \(\mu\)Ci) of HMM-\(^{14}\)C. Patient 2 received 93 mg (81.9 \(\mu\)Ci) of HMM-\(^{14}\)C together with 112 mg nonradioactive HMM. Patients were fitted with face masks (30), and their expired breath was monitored for \(^{14}\)CO\(_2\) by bubbling it through a solution consisting of 1 part redistilled ethanoleamine and 4 parts methanol (2). Aliquots of these ethanoleamine solutions were taken for counting. These \(^{14}\)CO\(_2\) collections were made during the 1st 54 min of each hr for the 1st 6 hr after the administration of the HMM-methyl-\(^{14}\)C. Twenty-four-hr urine and fecal samples were collected from patients for 3 days. Aliquots of the urine samples were taken for counting, and the samples then treated as described (11). Fecal samples were frozen until analysis; after thawing, they were homogenized in water and digested with nitric acid (27, 28). After digestion, an aliquot was counted by liquid scintillation. Plasma samples were centrifuged, protein was precipitated with sulfosalicylic acid (31), and an aliquot of the plasma was counted.

Sprague-Dawley male rats (Sprague-Dawley, Inc., Madison, Wis.) weighing 180 to 250 g were used throughout the experiments. Four rats were each given 1 ml of HMM-\(^{14}\)C solution containing 24 mg of unlabeled HMM and 1 mg of HMM-\(^{14}\)C (2.04 \(\times\) \(10^4\) dpm) by stomach tube. They were immediately placed in glass metabolism cages, where their \(^{14}\)CO\(_2\), urine, and feces were collected for 72 hr. Three rats were also given 1 ml of the HMM-\(^{14}\)C solution, except that they received it i.p. They were also placed in metabolism cages for 72 hr. The \(^{14}\)CO\(_2\) was collected by bubbling through ethanoleamine: methanol solutions, and these \(^{14}\)CO\(_2\) trapping solutions were changed and counted at 1, 3, 6, 12, and every 12 hr thereafter. For urine, the 24-hr samples were acidified to 0.1 N with HCl and applied to 7-cm resin beds of Dowex 1 (Cl\(^{-}\)) and rinsed with 100 ml 0.1 N HCl. The urine was acidified (0.1 N) with HCl and applied to 7-cm resin beds of Dowex 50W (H\(^{+}\)) and the columns were rinsed with 200 ml of 2.4 N HCl. The 2.4 N effluents were discarded, and then the columns were treated with 200 ml of 6 N HCl. These 6 N fractions were evaporated to dryness in vacuum, and the residues were treated with two 2-ml portions of concentrated NH\(_4\)OH and freeze dried after each treatment. The freeze-dried material was dissolved in 0.5 ml of 2-methoxyethanol, and the samples were then ready for gas chromatography.

A Tracor MT-220 gas chromatograph equipped with an alkali flame ionization detector was used. The detector was modified through the use of an RbSCvCsCl (3:1) pellet placed over the exit tip of the detector (1). A 60-cm glass column (4-mm inside diameter) containing 15% DEGS on Chromosorb W (60 to 80 mesh) was used. The column was well conditioned at 230\(^\circ\). The carrier gas was nitrogen (120 ml/min); hydrogen (40 ml/min) and air (275 ml/min) were used for the detector. Column temperature was programmed at 9\(^\circ\)/min from 150 to 245\(^\circ\) and held for 4 min.

For the gas chromatography: mass spectrometry experiments, a Hewlett-Packard 5700A gas chromatograph coupled to a Hewlett-Packard 5930 mass spectrometer system was used with a 70-cm aluminum column (3-mm inside diameter) packed with 15% DEGS on Chromosorb W. Helium was used as a carrier gas (50 ml/min), and the column temperature was 240\(^\circ\). Mass spectra were recorded at 70 eV and 30 ma trap current with the transfer lines at 240\(^\circ\) and source at 200\(^\circ\).

Test for Alkylating Activity. Solutions of HMM, the other methylmelamines, TEM, and appropriate blanks...
were assayed for alkylating activity using the NBP test (18). To a tube were added 2 ml of 0.25 mM solutions of the test compounds in methanol, 1 ml of 0.05 M potassium hydrogen phthalate buffer (pH 4.2), and 1 ml of 5% NBP solution (7). The tubes were placed in a 70° water bath for 2 hr and then transferred to an ice bath. After addition of methanol to a total volume of 5 ml, 3 ml of 0.01 N KOH in 80% methanol were added. The tubes were mixed and read immediately in a Bausch and Lomb Spectronic 20 spectrophotometer at 600 nm. Solutions of the methylmelamines equimolar in methyl groups to the 0.25 mM HMM solution were also assayed in the same manner.

RESULTS

Distribution of HMM-Methyl-14C in Humans and Rats. Recovery of radioactivity in CO2, urine, and feces after administration of HMM-14C to 2 cancer patients is shown in Table 1. Analysis of plasma levels of radioactivity 1 hr after administration revealed plasma levels of 3480 and 2680 dpm/ml for Patients 1 and 2. Radioactivity levels in plasma dropped steadily, but at 24 hr the plasma still contained 440 and 320 dpm/ml, respectively.

Recovery of excreted radioactivity after administration of HMM-14C to rats is shown in Table 2. The results of a study to determine the fate of radioactivity in various tissues of the rat after p.o. administration of HMM-14C are shown in Chart 1. There were no tissues which showed an especially high localization of radioactivity.

<table>
<thead>
<tr>
<th>Table 1</th>
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<tr>
<td>Recovery of radioactivity after administration of HMM-methyl-14C to 2 cancer patients</td>
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<tr>
<td>% of total dose of radioactivity</td>
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<tr>
<td>CO2</td>
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<tr>
<td>Patient 1*</td>
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<td>Patient 2*</td>
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<td>* Received 110 mg (219 x 10^4 dpm) HMM p.o.</td>
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<tr>
<td>* Received 205 mg (182 x 10^4 dpm) HMM p.o.</td>
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Urinary Excretion of HMM Metabolites. The urinary excretion patterns of HMM and its metabolites were examined to determine whether the metabolism of HMM in rats was similar to that observed in humans. Stepwise elution on ion-exchange columns (10) was performed on urines of rats that had received HMM-14C, and the fractions were assayed for radioactivity. When the acidified urines (0.1 N) were added to Dowex 1 (Cl-) columns and eluted with 0.1 N HCl, 98% of the radioactivity was recovered. This 0.1 N eluate was applied to Dowex 50W (H+) columns and eluted with stepwise gradients of HCl. The normality of each step of HCl and the recovery of radioactivity in that fraction are as follows: 0.1 N, 29%; 0.5 N, 3%; 1.0 N, 1%; 2.4 N, 2%, and 6 N, 55%. The 6 N fraction with 55% of the activity also contained all the UV-absorbing metabolites.

Gradient elution chromatography (11) was carried out on the 6 N fraction obtained from the stepwise elution. The effluent was monitored for UV absorption, and 4-ml fractions were assayed for radioactivity. Radioactive peaks coincided almost exactly with the spectrophotometric peaks (see Chart 2) except that Peak I contained very little radioactivity. A comparison of the UV absorption of the typical urinary excretion patterns of humans (11)
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and of rats (Chart 2) showed qualitative and quantitative similarities. Cochromatography of corresponding metabolite peaks from the urines of humans and rats confirmed that the peaks represented the same compounds in both species. Compounds II and III were most abundant in both rat and human urines.

Identification of Urinary Metabolites. Gas chromatographic analysis of rat urines showed 4 major urinary metabolite peaks plus 3 minor peaks (see Chart 3). Injection of a standard solution containing melamine and the 9 methylmelamine homologs gave the chromatogram shown in Chart 4. The retention times of the major urinary metabolites were found to be identical to those of known samples of \( N^2, N^4, N^6 \)-trimethylmelamine, \( N^2, N^4 \)-dimethylmelamine, monomethylmelamine, and melamine. The minor peaks had retention times similar to pentamethylmelamine, \( N^2, N^4, N^6 \)-tetramethylmelamine, and \( N^2, N^4, N^6 \)-trimethylmelamine.

Mass spectra of the rat urinary metabolites of HMM were obtained as they eluted from the gas chromatograph, and these spectra were then compared to mass spectra of known melamine and methylmelamine compounds. This gave positive identification of the major urinary metabolites (Charts 2 and 3) as \( N^2, N^4, N^6 \)-trimethylmelamine (IV), \( N^2, N^4 \)-dimethylmelamine (III), monomethylmelamine (II), and melamine (I). The minor urinary metabolites were identified as pentamethylmelamine, \( N^2, N^4, N^6 \)-tetramethylmelamine, and \( N^2, N^4, N^6 \)-trimethylmelamine.

Test for Alkylating Activity. The NBP alkylation test (7, 18) was performed on equimolar solutions of melamine, all methylmelamines, and TEM. The test was also repeated on samples of all the methylmelamines which were equimolar in methyl groups to HMM. The absorbance values ranged from 0.00 to 0.02 for all the samples except TEM, which had an absorbance of 0.68. Thus, TEM was the only compound to demonstrate significant alkylating activity in this test.

DISCUSSION

Structural similarities between HMM and TEM make it of interest to compare the metabolism of these 2 chemicals. Smith et al. (33) reported that the metabolism of TEM containing a \( ^{14} \text{C} \)-label in the ethylenimino moiety provided "comparatively little radioactivity" in the exhaled CO\(_2\) of mice. In contrast, 16 to 33% of the radioactivity from administration of HMM-methyl-\( ^{14} \text{C} \) to rats was found as \( ^{14} \text{CO}_2 \). Nadkarni et al. (26) reported that in mice TEM was metabolized, and 72 to 88% was excreted in urine as cyanuric acid. The identification of the major human and rat urinary metabolites of HMM as melamine compounds shows that HMM differs metabolically from TEM.

Quantitatively, the amounts of HMM metabolites were determined (13) from radioactivity measurements of metabolite peaks separated by gradient elution ion-exchange chromatography. In rats, at least 64% of the HMM administered could be accounted for as methylmelamines in the 1st 24-hr urine; 4% as \( N^2, N^4, N^6 \)-trimethylmelamine, 33% as \( N^2, N^4 \)-dimethylmelamine, and 27% as monomethylmelamine. This does not include melamine, which could not be determined from radioactivity measurements because of the absence of any methyl-\( ^{14} \text{C} \).
groups. However, monitoring UV absorption during gradient elution chromatography gave an estimate of the amount of melamine (4%), since the UV absorption is very similar for these melamine compounds.

HMM is metabolically very active in rats as evidenced by the fact that no unchanged HMM could be detected by ion exchange or gas chromatography. The identification of urinary metabolites led to the formulation of a metabolic pathway for HMM (see Chart 5) which follows the solid arrows. It is interesting to note the presence in the urine of major quantities of only 1 of 2 possible isomers of tri- and tetramethylmelamine. From the pattern of metabolites observed, it appears that a dimethylamino group may be N-demethylated preferentially to a monomethylamino group (15).

These experiments demonstrated that in rats greater than 64% of HMM was excreted as metabolites containing an intact s-triazine ring, suggesting the metabolic stability of s-triazines in mammalian systems (4, 5). In the stepwise gradient elution of urines on ion-exchange columns, 29% of the radioactivity was found in the 0.1 N HCl fraction, but this fraction did not contain UV-absorbing material. The metabolites in this fraction were not identified.

The metabolism of HMM in humans is very similar to that in rats. HMM was N-demethylated, and 9% of the radioactivity from HMM-methyl-14C was excreted as 14CO2 within 6 hr. The major urinary metabolites were the same as those observed in rats. The peak plasma level of radioactivity in patients occurring 1 hr after administration of HMM-methyl-14C differs from the peak level of UV-absorbing metabolites (11) which occurs at 2 to 3 hr. This discrepancy may be explained by the metabolism of HMM which involves N-demethylation; there could be an increase in the concentration of UV-absorbing metabolites, together with a decrease in plasma radioactivity, as a result of an increase in the concentration of metabolites containing fewer methyl-14C groups.

HMM is an effective chemosterilant for house flies (14), and the metabolism of HMM in male house flies has been studied by Chang et al. (13). They reported that the principal metabolic transformation of HMM in flies involved N-demethylation and identified pentamethylmelamine, N2,N2,N4,N6-tetramethylmelamine, N2,N4,N6-trimethylmelamine, and N2,N3,N4-trimethylmelamine as metabolites. They were also unable to detect any HMM or N2,N2,N4,N6-tetramethylmelamine. Thus, it would appear that similarities exist between humans, rats, and flies in their metabolism of HMM.

The mechanism of action for the antineoplastic properties of HMM is unknown. For cancer chemotherapy, the dosage level of HMM is approximately 200 times as great as that of TEM. Philips and Thiersch (29) also reported that the 50% lethal dose of HMM in rats and mice was approximately 100 times greater than for TEM. These facts suggest that HMM has a different mechanism of action than its structural analog, TEM, which is an alkylating agent (33). Bořkovč and others (9, 13, 17, 23) have attempted to learn the basis for the sterilizing activity of HMM in flies, and they have stated (9, 23) that HMM does not act as an alkylating agent in the generally accepted meaning of the term. The negative results of HMM and its metabolites with the NBP alkylation test in these experiments, while TEM gave a positive result, also suggest that HMM should not be classified as an alkylating agent at this time, contrary to previous suggestions (36).

However, the clinical spectrum and toxicity of HMM resemble those of alkylating agents (8). Thus, the possibility that intermediates in the metabolism of HMM or unidentified metabolites might possess alkylating activity cannot be dismissed. Cyclophosphamide, for example, shows very little reaction with NBP (32), but its active form is believed to be an alkylator.

There is evidence that certain s-triazines can function as pyrimidine antimetabolites (34, 35). Heere and Donnelly (19) reported that HMM exhibited greater inhibition of uptake of DNA and RNA precursors than of protein precursors in Ehrlich ascites tumor cells in vitro. Some diaminodihydro-s-triazines act as folic acid antagonists (3). Thus, it is evident that further experiments are required to determine the mechanism of action of the antineoplastic properties of HMM.

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