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Pharmacokinetics and metabolism of hexamethylmelamine in mice after IP administration

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Summary. The pharmacokinetics of hexamethylmelamine (HMM) and its first metabolite (hydroxymethylpentamethylmelamine: HMPMM) following IP bolus dose of 200 mg/kg were studied in mice. The drug concentrations were determined by a sensitive reversed-phase HPLC assay. Thus, for the first time, HMM major hydroxylated and demethylated metabolite plasma levels canbedetermined at the same time. Pharmacokinetic data were analyzed by an original method using a nonlinear cost function minimized by a simplex algorithm. An important property of this computer program is that convergence is ensured in contrast to linear or nonlinear least-square regression analysis, which leads to lack of convergence or to false convergence. Both HMM and HMPMM data fit a one-compartment open model. The parameters obtained indicate that the parent drug would probably be rapidly and completely transformed by the human body into HMPMM.

Interproduction

Hexamethylmelamine (HMM) is an investigational antitumor agent with demonstrated activity both as a single agent and in combination with other drugs against several types of cancer, including ovarian carcinoma, lymphomas, and small cell carcinoma of the lung [9, 20]. The mechanism of action of HMM has not yet been elucidated. This drug originally was thought to be an alkylating agent similar to triethylenemelamine, but failure of the in vitro nitrobenzyl pyridine (NBP) test led Worzalla et al. to conclude that alkylation is not the mechanism responsible for its antitumor effect [25]. Some authors have mentioned that HMM demonstrated activity in patients resistant to alkylating agent therapy [9]. Nevertheless, it has recently been demonstrated in vivo [16] and in vitro [5] that metabolic intermediates could interact covalently with macromolecules.

It has also been demonstrated that the drug itself is cytotoxic in vitro only after prolonged exposure to cells [12]. However, one of its major methylol metabolites, detected in vivo as well as in vitro, hydroxymethylpentamethylmelamine (HMPMM), is highly cytotoxic when incubated with human and murine tumor cell lines [12, 23]. Thus, there is good evidence that HMM requires metabolic activation to develop antitumor activity. The pharmacokinetics and metabolism of HMM have been studied separately in several species [10, 11, 19, 24–26]. In this paper, the results of a study are reported in which, for the first time, HMM major hydroxylated (HMPMM) and demethylated (pentamethylmelamine PMM, 2, 2, 4, 6 tetramethylmelamine 2, 2, 4, 6 tetr MM) metabolite plasma levels were detected at the Same time by a HPLC method. The pharmacokinetic data obtained were analyzed by means of the original version of the simplex method [1].

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Materials and methods

Chemicals HMM, PMM, 2, 2, 4, 6 tetrMM, and HMPMM were kindly provided by Simon Langdon (Aston University, Department of Pharmacy, Great Britain). All reagents were analytical grade (Merck) except for acetonitrile (Rathburn chemicals LTD, Scotland; HPLC grades).

Animal studies. Groups of six male CDF_1 (Balb/C × DBA/-2) mice weighing 25 ± 2 g were administered 200 mg HMM/kg in 1 ml Klucel suspension intraperitonally. Animals were anaesthetized with diethyl ether at various times after drug administration, and blood samples were collected by cutting the femoral vein. Blood was placed in heparanized glass tubes and kept on ice. Plasma was prepared by centrifuging at 2000 rpm for 10 min at 4 °C; 0.6 ml CH_3CN was added to 0.2 ml plasma in a centrifuge tube. The mixture was vortex mixed for 10 s, then centrifugated at about 4500 rpm for 10 min (4 °C) to pellet the precipitated proteins.

Aliquots (50–100 µl) of the supernatant were analyzed by chromatography.

Metabolite determination. Estimation of N-methyl and N-methyolmelamines in plasma was made using a Perkin-Elmer 601 liquid chromatograph equipped with a Perkin-Elmer LC 55-S variable wavelength ultraviolet detector, set at 220 nm, and a Rheodyne 7105 injector. Separations were achieved on a 6.5 µm reversed-phase Merck Hibar Lichrocart RP_{18} column (4.0 mm × 250 mm), using 0.01 M phosphate buffer (NaH_2PO_4–Na_2HPO_4 1/1) and acetonitrile (68:32). The pH of this mixture was first adjusted to 4, with 85% H_3PO_4 and then to 7.5 with *n*-propylamine. The flow rate was 1.5 ml/min. The column and mobile phase temperature were maintained at 20 °C with a water bath [13]. Retention time data were obtained directly from chromatograms as recorded on a potentiometer recorder (1 mV full scale). Peak height was used for quantification.

Data analysis

The pharmacokinetic parameters were obtained by a modified FADHA program [1]: FADHA II. In contrast to FADHA analyses, the FADHA II program data obtained for the drug administered and its first metabolite at the Same time by means of an algorithm based on the simplex method [7, 22] that minimizes nonlinear cost function. This function is expressed by Eq. (1) for the drug administered (HMM).

$$F_p(P_1) = \sum_{k=1}^{N_p} \left(\frac{\Delta C_{ij}}{\sigma c_{ij}}\right)^2 + \left(\frac{\Delta t_{ij}}{\sigma t_{ij}}\right)^2 \quad (1)$$

In this equation N_P is the number of experimental points, ΔC_{IJ} is the difference between the observed (C_I) and estimated (C_{IJ}) plasma concentrations at time T_I for experiment J, σC_{IJ} is the estimated error on the C_{IJ} determination, ΔT_{IJ} is the difference between the estimated (T_{IJ}) and observed T_I times for the experiment J at time T_I, σT_{IJ} is the estimated error on T_I for all J, and P_1 is an estimation of the administered drug (HMM) parameters. Equation (2) is the expression of the nonlinear cost function for the metabolite HMPMM.

$$F_m(P_2) = \sum_{k=1}^{N_m} \left(\frac{\Delta C_{ij}}{\sigma C_{ij}}\right)^2 + \left(\frac{\Delta t_{ij}}{\sigma t_{ij}}\right)^2 \quad (2)$$

N_m is the number of experimental points for the metabolite (HMPMM) P_2 is an estimation of the metabolite (HMPMM) parameters. The FADHA II program uses a method that minimizes the cost function expressed by Eq. (3).

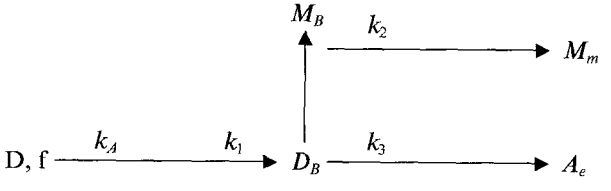
$$F(P_1 \cdot P_2) = F_P(P_1) + F_m(P_2) \quad (3)$$

As can be seen, this last equation takes Eqs. (1) and (2) into account. The plasma concentration versus time curves can be determined with Eq. (4) and Eq. (5) for HMM and HMPMM, respectively.

$$C_p = \frac{D \cdot f}{Vd} \cdot k_A \left\{\frac{\exp[-K_A(t-t_0)]}{K-k_A} + \frac{\exp[-K(t-t_0)]}{k_A-K}\right\} \quad (4)$$

$$M_p = \frac{D \cdot f}{Vd} \cdot k_A \cdot k_1 \left\{\frac{\exp[-K_A(t-t_0)]}{(K-k_A)(k_2-k_1)} + \frac{\exp[-K(t-t_0)]}{(k_A-K)(k_2-K)} + \frac{\exp[-K_2(t-t_0)]}{(k_A-k_2)(K-k_2)}\right\} \quad (5)$$

The model used in this study is then as follows:



where: D = dose size administered (μg); F = fraction of administered dose that is absorbed; K_A = absorption rate constant (min^{-1}); D_B = amount drug in body (μg); K_1 = formation rate constant of metabolite (min^{-1}); K_2 = elimination rate constant of metabolite (min^{-1}); K_3 = elimination rate constant of unchanged drug (min^{-1}); M_M = amount of metabolite excreted at time T (μg); A_O = amount of drug excreted at time T (μg); K = overall elimination constant ($K_1 + K_3$).

Some of the advantages of using the simplex algorithm have abreadly been reported [1]. They include:

- Guaranteed convergence to the absolute minimum, even if there are relatively few experimental data.
- Convergence does not depend on the choice of the starting values of the parameters.
- This algorithm takes all of the possible observation errors into account (analytical error, imprecise time, and biological intraindividual and/or interindividual variability).
- Not only the means, but all experimental data were analyzed.

Results

Standard curves, linearity range and detection limit for methyl-melamines

The peak height was platted; the mean of triplicate injections of calibration standard solutions versus the amount of each methylmelamine injected was linear between 20 and 400 ng/injection, with a mean negligible intercept; the mean slope of the linear portion of the line was also determined. The regression data and correlation coefficients are listed in Table 1. In all cases, good linearity was observed. The detection limits calculated as three times the noise level for each melamine are given in the last column of this table.

Recovery studies

The recovery of melamine derivatives was measured by comparison of the peak height of standards dissolved during chromatography to the peak obtained from standards extracted from mice plasma according to the method described below. Table 2 shows that this method led to recoveries of about 100% for all derivatives studied. We verified that there was neither melamine adsorption on the precipitated proteins nor volume variation due to this precipitation. The efficiency of the assay was assessed by quadruplicate assay of spiked plasma sample containing about 0.8 and 4 μg ml^{-1} melamine. Typical chromatograms observed after injection of plasma extracts from mice treated with HMM are shown in Fig. 1. No endoge-

Table 1. Standard curves, linearity range and detection limit for methylmelamines

Compounds	Standard curves				Detection limits (ng)
	Linearity range (ng)	$\gamma = a\chi + b$			
		b	a	r^2	
HMM	35–670	5.08	18.37	0.999	16.0
PMM	20–295	3.62	9.91	0.999	9.5
HMPMM	31–385	10.93	11.15	0.999	17.0
2246	22–340	6.20	9.77	0.999	12.0

γ = absorbance; χ = amount of methylmelamine injected; r^2 = correlation coefficient

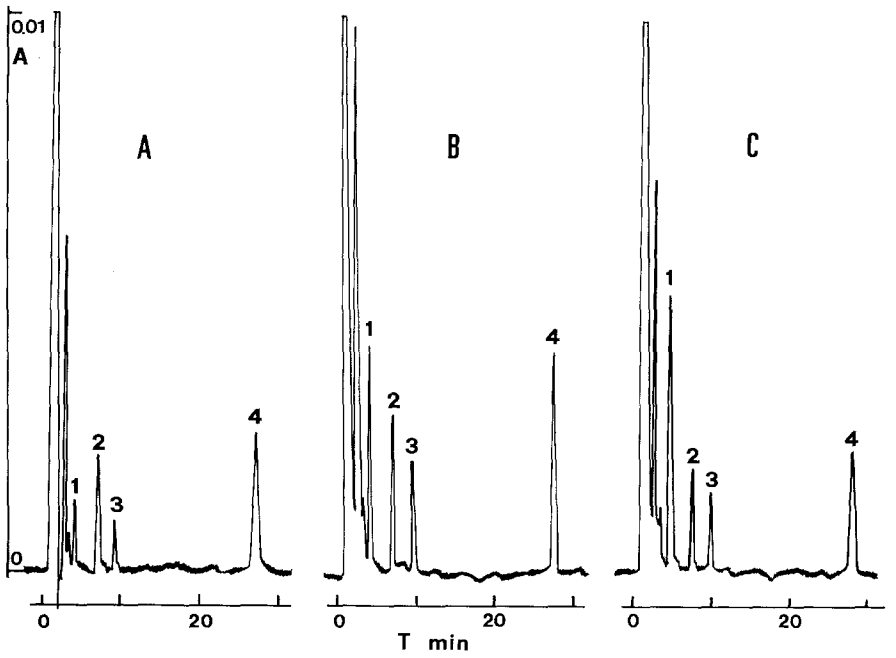


Fig. 1 A–C. Chromatograms of mice plasma 15 min **(A)**, 30 min **(B)**, 1 h **(C)**, after IP administration of 200 mg/kg of HMM. *1*: 2, 2, 4, 6 TetrMM; *2*: HMPMM; *3*: PMM; *4*: HMM. In each case, aliquots of 50 µl were injected

nous compounds with retention times corresponding to that of melamines were encountered.

Pharmacokinetics of HMM and its hydroxylated metabolite (HMPMM) in mice

After IP bolus injection of 200 mg/kg HMM in mice, plasma concentrations versus time data of this drug and its metabolite HMPMM were analyzed by means of the FADHA II program and then fitted to a one-compartment model with an extra compartment of administration for HMM.

In this model, the lag time for HMM and HMPMM was essentially the same (1.88 min). The HMM absorption rate constant (K_A) was 4.40 min^{-1} and its absorption half-time was 0.16 min. The metabolism of the parent drug was rapid as indicated by the plasma $T^K1/2$ value (43.55 min); the overall elimination constant (K) was 0.02 min^{-1}. The appearance of the first metabolite (HMPMM) was also rapid: its formation rate constant in the body (K_1) was 0.02 min^{-1} and the corresponding half-time ($T^{KJ}1/2$), 43.55 min. The elimination rate constant of this compound was 0.04 min^{-1} while its half-time ($t^{k_2}_{1/2}$) was 16.06 min. These results appear to be very important as they led us to assume that the parent drug would be immediately and completely transformed into HMPMM in the human body. The rate constant of elimination of the unchanged drug is in effect equal to zero ($K_3 = K - K_1 = 0$). The area under the HMM plasma concentration vs time curve from zero to infinity ($AUC_{0\to\infty}$) is 1296.03 min µg ml^{-1} and about one-third of this value for HMPMM: 477.85 min µg ml^{-1}. The plasmatic maximum concentrations (C_{max}) were 4.25 and 20.20 µg ml^{-1} for HMPMM and HMM, respectively. This maximal concentration was rapidly observed for the last

Table 2. Determination of methylmelamines in mice plasma. Recovery study

Compound	Added to plasma (µg ml^{-1})	Recovery (%)		
		Mean[a]	Range	RSD[b] (%)
HMM	3.8	99.2	95.6–101.4	2.8
	1.9	99.2	95.7–106.5	5.5
PMM	2.5	99.8	96.5–102.0	2.1
	1.25	99.3	95.7–106.2	5.3
HMPMM	1.7	99.5	94.6–105.2	5.3
	0.85	98.1	95.9–102.0	2.8
2246 TetrMM	1.7	98.3	97.3–100.5	1.4
	0.85	97.7	92.3– 99.5	3.1

[a] Calculated from four measurements
[b] Relative standard deviation (%)

Table 3. Pharmacokinetic parameters of hexamethylmelamine and its first metabolite (HMPMM) after IP injection of 200 mg/kg in mice (one-compartment open model)

Compound	Pharmacokinetic parameters							
HMM	Lag time (min)	k_A (min^{-1})	$t^{k_A}_{1/2}$ (min)	K (min^{-1})	$t_{1/2}{}^K$ (min)	$AUC_{0\infty}$ (µiv µg/ml^{-1})	t_{max} (min)	C_{max} (µg/ml^{-1})
	1.88	4.40	0.16	0.02	43.55	1296	3.16	20.20
HMPMM	Lag time (min)	k_1 (min^{-1})	$t^{k_1}_{1/2}$ (min)	k_2 (min^{-1})	$t^{k_2}_{1/2}$ (min)	$AUC_{0\infty}$ (µiv µg/ml^{-1})	t_{max} (min)	C_{max} (µg/ml^{-1})
	1.88	0.02	43.55	0.04	16.06	477	38.73	4.25

Table 4. Concentration data versus time in mice

	Time (min)					
	2	15	30	60	120	180
2,2,4,6 tetrMM	Not	1.50	3.06	9.34	2.69	2.11
(μg/ml^{-1}	detected	1.53	4.87	6.25	4.62	1.43
		1.34	3.26	6.37	4.49	2.79
		1.08	3.00	3.94	3.17	1.64
		1.57	3.57	5.61	3.72	2.58
		1.16	3.32	–	–	–
		1.58	–	–	–	–
PMM	Not	2.68	2.63	4.26	0.86	Not
(μg/ml^{-1}	detected	1.67	3.62	2.82	1.13	detected
		2.95	1.50	2.64	1.18	
		1.45	2.91	2.26	1.33	
		2.41	2.46	2.92	1.14	
		1.97	2.79	–	1.44	
HMPMM		5.27	4.50	4.00	1.10	7.23
(μg/ml^{-1}		4.20	4.61	3.07	1.24	0.93
		6.56	3.72	2.54	1.77	1.08
		5.64	4.40	3.73	2.13	1.38
		8.29	5.01	4.82	2.13	0.78
		5.80	4.82	–	2.00	–
		5.22	–	–	–	–
HMM		17.66	6.77	9.18	2.09	1.54
(μg/ml^{-1}		24.94	13.61	7.32	3.54	2.09
		30.38	10.40	6.97	5.30	2.27
		17.12	15.84	9.48	5.07	3.01
		38.11	15.86	13.16	4.87	2.45

compound (T_{max} = 3.16 min) but more delayed for HMPMM (T_{max} = 38.73 min). These pharmacokinetic parameters are listed in Table 3. Table 4 shows the concentration data versus time for all the compounds studied.

Figure 2 shows the monophasic disappearance of both HMM and HMPMM from mice plasma following IP administration of the dose.

Discussion

The greatest difficulty generally encountered by investigators when studying pharmacokinetic parameters is the elaboration of a very sensitive and specific analytical method to determine traces of drug in biological fluids. So far mel-

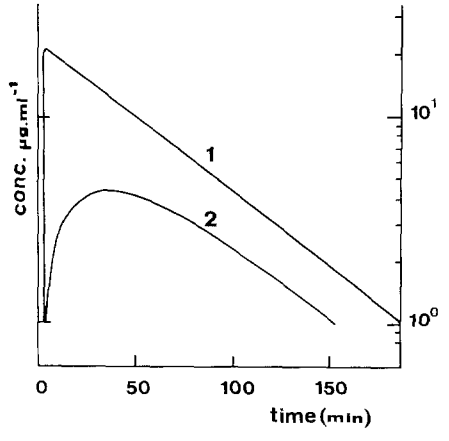


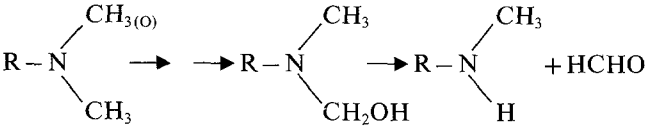
Fig. 2. The pharmacokinetics of HMM *(1)* and HMPMM *(2)* in mice following IP administration of 200 mg/kg HMM. Lines were fitted as described in the Materials and methods section under data analysis

amine derivatives have been estimated in biological samples by gas chromatography [2, 11, 14, 19] or thin-layer and high-performance liquid chromatography [6, 17], but these methods are not suitable for separating and quantifying HMM and its main demethylated and hydroxylated metabolites at the same time. These assays often induce breakdown of the N-methylolmelamines to methylmelamines and give an artificially high estimation of the concentrations of the latter compounds; the total N-methylolmelamine content of samples then must be assayed via the formaldehyde generated in the Nash method [3, 16]. We were able to develop a sensitive nondestructive HPLC assay and a safe method of extraction, which presented an alternative method of study way the HMM metabolism and its pharmacokinetics.

The pharmacokinetic parameters formed in our study showed that HMM is rapidly absorbed from its compartment of administration and that its rapid metabolism led to pharmacological intermediates.

The pathway of HMM metabolism has so for been thought to be N-demethylation, but some authors have postulated the presence of a stable carbinolamine intermediate: HMPMM [4, 5, 9, 13]. Our findings support this interpretation. Under the chromatographic conditions described, three metabolites can be determined in the same sample: HMPMM; PMM; 2, 2, 4, 6 tetr MM.

In most demethylation metabolism pathways, the initial oxidation product decomposes to the corresponding aldehyde and dealkylated amines [21, 15]. However, it has been proposed that the substituents, adjacent to the nitrogen atom, that delocalize the lone pair of nitrogen electrons can stabilize carbinolamines [5]. Stable carbinolamines have also been isolated as metabolites to a number of drugs, including benzamides and the antitumor agent DTIC [4, 18]. Our results show that the first metabolite, HMPMM, appeared in mouse plasma at almost the same time as HMM. A lag time of 1.88 min was observed for these two compounds; then PMM and 2, 2, 4, 6 tetr MM were detected in turn. From the detection sequence of metabolites in plasma, it was possible to establish a definitive metabolic pathway for HMM:



The half-life of HMPMM is about one-third the half-life of HMM. The area of HMPMM under the plasma concentrations versus time curve ($AUC_{O\to\infty}$) is less than one-third of the HMM $AUC_{O\to\infty}$ value. The fact that HMPMM has a rather high biodisposability is an interesting phenomenon that night explain HMM activity.

The direct in vitro cytotoxic effect of N-hydroxymethyl compounds has been reported as belonging to a wide variety of structures [17], and these drugs are chemically characterized by their ability to form aminomethylate nucleophiles. It has also been demonstrated that HMPMM binds covalently to calf thymus DNA and, to a lesser extent, to microsomal protein [5].

Moreover, HMM is known to be a better clinical antitumor drug than PMM, which is the second metabolite of HMM. Pharmacokinetic analysis of plasma concentration by means of the simplex method for HMM and HMPMM

led us to consider the entire human body as a one-compartment model. The result may indicate that these drugs are rapidly distributed between the plasma and tissue, including tumoral tissue, when entering the systemic circulation. A systematic investigation of the pharmacokinetics, distribution and metabolism of HMM, especially in tumors, Blatt 11 be necessary to understand the pharmacological properties completely.

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