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DETERMINATION OF HEXAMETHYLMELAMINE AND METABOLITES IN PLASMA OR SERUM BY GAS—LIQUID CHROMATOGRAPHY WITH A NITROGEN-SENSITIVE DETECTOR

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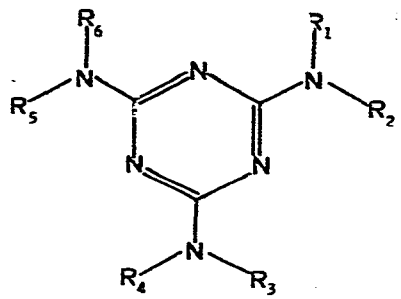
SUMMARY

A gas chromatographic method for the quantitative determination of hexamethylmelamine (HMM) and five of its metabolites in plasma (or serum) is described. After adjustment of the pH of the plasma sample to about 9.5, the compounds are extracted with chloroform containing 5% of isopropanol. Amyl alcohol is added to the extract, which is then evaporated until a small volume remains. An aliquot of this solution is injected into a gas chromatograph equipped with a nitrogen—phosphorus flame ionisation detector. Separation of the methylmelamines is achieved with a 10% Carbowax 20M—2% KOH column. By programming the oven temperature unnecessarily long retention times are avoided.

Using 1 ml of plasma, concentrations as low as 5 ng/ml of HMM and its metabolites can be quantitated. The method has been applied to the determination of HMM and metabolites in plasma of patients who received oral doses of HMM.

INTRODUCTION

Hexamethylmelamine (HMM) is an orally administered anti-tumor agent, which is active in different human malignancies and is used in the treatment of ovarian adenocarcinoma [1—4]. In vivo it is quickly converted into demethylated metabolites [5, 6], of which pentamethylmelamine (PMM) is also considered for therapeutic use [7]. With the exception of N^2,N^2,N^4,N^4-tetramethylmelamine ($TeMM_1$) all of the possible demethylated analogues of HMM have been recovered from urine samples of patients treated with HMM [6] (Fig. 1).



	Compound	R_1	R_2	R_3	R_4	R_5	R_6
1	Hexamethylmelamine (HMM)	CH_3	CH_3	CH_3	CH_3	CH_3	CH_3
2	Pentamethylmelamine (PMM)	CH_3	CH_3	CH_3	CH_3	CH_3	H
3	N^2,N^2,N^4,N^4-Tetramethylmelamine ($TeMM_1$)	CH_3	CH_3	CH_3	CH_3	H	H
4	N^2,N^2,N^4,N^6-Tetramethylmelamine ($TeMM_2$)	CH_3	CH_3	CH_3	H	CH_3	H
5	N^2,N^2,N^4-Trimethylmelamine ($TriMM_1$)	CH_3	CH_3	CH_3	H	H	H
6	N^2,N^4,N^6-Trimethylmelamine ($TriMM_2$)	CH_3	H	CH_3	H	CH_3	H
7	N^2,N^2-Dimethylmelamine (DMM_1)	CH_3	CH_3	H	H	H	H
8	N^2,N^4-Dimethylmelamine (DMM_2)	CH_3	H	CH_3	H	H	H
9	Monomethylmelamine (MMM)	CH_3	H	H	H	H	H
10	Melamine (MA)	H	H	H	H	H	H

Fig. 1. Structural formulas of the methylmelamines.

In a recent study the peak plasma concentrations of HMM obtained after oral administration of the drug were found to differ markedly between patients [8]. Varying (incomplete) absorption from the gut or differences in metabolic rates (first pass effect) could account for this. The metabolism of HMM might be influenced by other drugs, such as 5-fluorouracil, methotrexate and cyclophosphamide, when co-administered with HMM in combination chemotherapy. The aim of the present investigation was to develop a method for the quantitative determination of HMM and a number of its metabolites in plasma. The metabolic conversion of HMM can then be followed after administration of the drug in various dosage forms, and in different therapy schemes.

A number of chromatographic methods for the analysis of HMM and/or the other methylmelamines have been described [5, 6, 8—16]. In recent studies [8, 10], the determination of HMM and of PMM in plasma by gas chromatography with nitrogen—phosphorus detection was reported. In the present study we also used a gas chromatograph equipped with a nitrogen-sensitive detector. We were

able to quantitate HMM and five of its metabolites in concentrations as low as 5 ng/ml plasma. The method was applied to plasma samples from patients following oral administration of HMM.

MATERIALS AND METHODS

HMM was obtained from Ofichem (Gieten, The Netherlands; batch No. 790205). The metabolites of HMM used in this investigation were a gift of Dr. D.E.V. Wilman (Institute of Cancer Research, Royal Cancer Hospital, London, Great Britain). Chloroform and methanol (both nanograde quality) were from Mallinckrodt (St. Louis, Mo., U.S.A.). Isopropanol, zur Analyse (Merck, Darmstadt, G.F.R.) and amyl alcohol (Brocacef, Maarssen, The Netherlands) were distilled from glass prior to use. Sodium hydroxide (zur Analyse, Merck) and pethidine hydrochloride (Brocacef) were used without further purification. Centrifuge tubes of 7 ml capacity, with glass stoppers and the conical ends drawn to a fine point, were used.

Gas chromatography

A Hewlett-Packard Model 5710A gas chromatograph equipped with a Model 18789A dual nitrogen—phosporus flame ionisation detector was used. The glass column (120 cm × 1.9 mm I.D.) was silanized and packed with 10% Carbowax 20M—2% KOH on 80—100 mesh Chromosorb W AW (Chrompack, Middelburg, The Netherlands) and conditioned overnight at 230°.

The operating conditions were: injection port temperature, 285°; detector temperature, 360°; carrier gas (nitrogen) flow-rate, 30 ml/min; hydrogen flow-rate, 3.6 ml/min; air flow-rate, 55 ml/min. The detector voltage (d.c.) was set at about 14 V.

An oven temperature program was maintained following each injection: 2 min isothermal heating at 194°, then 4°/min from 194—230°, and 8 min isothermal heating at 230°. A 4-min period of cooling and stabilizing was maintained between injections.

Procedure

A 1.0-ml volume of plasma or serum (stored at −20° until analysis) was transferred to a centrifuge tube and mixed with 100 µl methanol and 10 µl 2.0 *N* sodium hydroxide (resulting pH 9.5—9.7). After the addition of 3.0 ml chloroform—isopropanol (95:5), containing 290 ng/ml pethidine hydrochloride as internal standard, the contents of the tube were shaken manually for 1 min. Following centrifugation (2500 *g*, 5 min) the upper aqueous layer was removed; the organic phase was transferred to a centrifuge tube and 50 µl amyl alcohol was added. After mixing, the solvent was removed under a stream of nitrogen until a volume of about 50 µl remained, of which 2 µl were injected into the gas chromatograph.

Calibration curve

Pooled serum samples (1 ml) were transferred to centrifuge tubes and spiked with 5—100 µl of a methanolic solution containing HMM (5.05 µg/ml), PMM (2.50 µg/ml), $TeMM_1$ (0.97 µg/ml), $TeMM_2$ (2.50 µg/ml), $TriMM_1$ (1.08 µg/ml)

and $TriMM_2$ (4.94 μg/ml). After the addition of methanol to a total amount of 100 μl, the samples were treated further as described under Procedure. Following chromatography the peak heights were measured and the peak-height ratios of HMM and its metabolites to the internal standard were plotted against the concentration in the samples.

Determination of the absolute recoveries of HMM and metabolites

To 1-ml pooled serum samples in centrifuge tubes, 100-μl portions of the methanolic solution described above were added. After addition of 10 μl 2 *N* sodium hydroxide and mixing, 3.0 ml chloroform—isopropanol (95:5) were added and the tubes were shaken manually for 1 min. Following centrifugation (2500 *g*, 5 min), the aqueous layer was removed and the organic phase transferred to a centrifuge tube. The solvent was blown off (nitrogen), taking care that the dried samples did not remain under the nitrogen stream.

The residues in the tubes were reconstituted in 50.0-μl methanol portions, containing 17.2 μg/ml pethidine hydrochloride as internal standard. Of this solution 2 μl were injected. From the resulting peak-height ratios (HMM and metabolites to the internal standard), and the peak-height ratios obtained after chromatography of a standard solution of HMM and its metabolites and pethidine hydrochloride, the absolute recoveries of each of the methylmelamines under investigation were calculated.

RESULTS AND DISCUSSION

The Carbowax—KOH stationary phase provides excellent chromatographic properties, not only to HMM and PMM as reported previously [10], but also to seven other methylmelamines and the internal standard, pethidine. The peaks of all compounds mentioned in Fig. 1 and of pethidine were completely separated under the prevailing conditions. The retention times are given in Table I.

TABLE I

RETENTION TIMES AND ABSOLUTE RECOVERIES FROM SERUM OF METHYLMELAMINES AND PETHIDINE

Compound*	Retention time (min)	Recovery (%)** ± S.D.
HMM	2.7	85.8 ± 4.3
PMM	4.8	82.9 ± 2.3
$TeMM_1$	6.6	85.3 ± 3.0
$TeMM_2$	7.6	84.6 ± 2.9
$TriMM_1$	9.6	83.8 ± 4.0
$TriMM_2$	10.8	74.4 ± 3.2
DMM_1	11.8	
MMM	13.1	
MM	20.9***	
Pethidine	3.8	

*The notation of the compounds is explained in Fig. 1; N^2,N^4-dimethylmelamine (DMM_2) was not available.

**The mean values and standard deviations (S.D.), obtained from 6 experiments, are given.

***The isothermal period at the end of the oven temperature program was extended to 12 min.

A chromatogram obtained after injection of the methanolic solution of HMM and five of its possible metabolites is shown in Fig. 2A.

Chromatograms of a control patient plasma sample and of one, taken from a patient 3 h after administration of an oral dose of 300 mg HMM, are shown in Fig. 2B and C, respectively. Peaks with retention times corresponding to HMM, PMM, $TeMM_2$, $TriMM_1$ and $TriMM_2$ were apparent in the chromatogram of the patient sample (Fig. 2C). Using a 2% OV-225—1% OV-17 column, the retention times of the relevant peaks in the chromatogram of the plasma sample were the same as those obtained after injection of a solution of HMM and its analogues onto the same column.

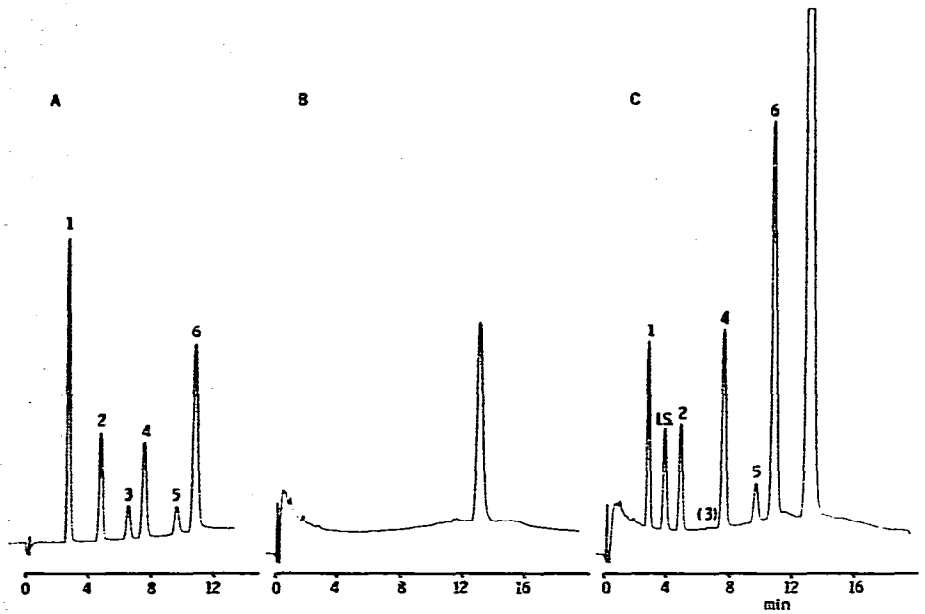


Fig. 2. Chromatograms obtained from (A) a methanolic solution of HMM and 5 demethylated analogues, (B) a control patient plasma sample, and (C) a patient plasma sample 3 h after the oral administration of 300 mg HMM. The peak numbers correspond to the compounds as denoted in Fig. 1 (I.S. = internal standard).

After extraction of aqueous solutions of DMM_1, MMM and MM, following the procedure described under Materials and methods, about 30% of DMM_1, 1% of MMM and no measurable amounts of MM were recovered. No significant peaks of MMM and MM could therefore be expected. The DMM_1 peak, if at all present, coincides with a large plasma peak.

In most chromatograms obtained from patient samples no $TeMM_1$ peak could be discerned. In some of the chromatograms a small peak appeared with the same retention time as the $TeMM_2$ peak. Assuming the corresponding compound to be $TeMM_1$, its concentration always remained under the 5 ng/ml plasma level.

Ames and Powis [10] stated that oven temperatures higher than 190° can not be used because of the limited stability of the stationary phase. The upper temperature limit of 230° in our investigation is, however, consistent with the

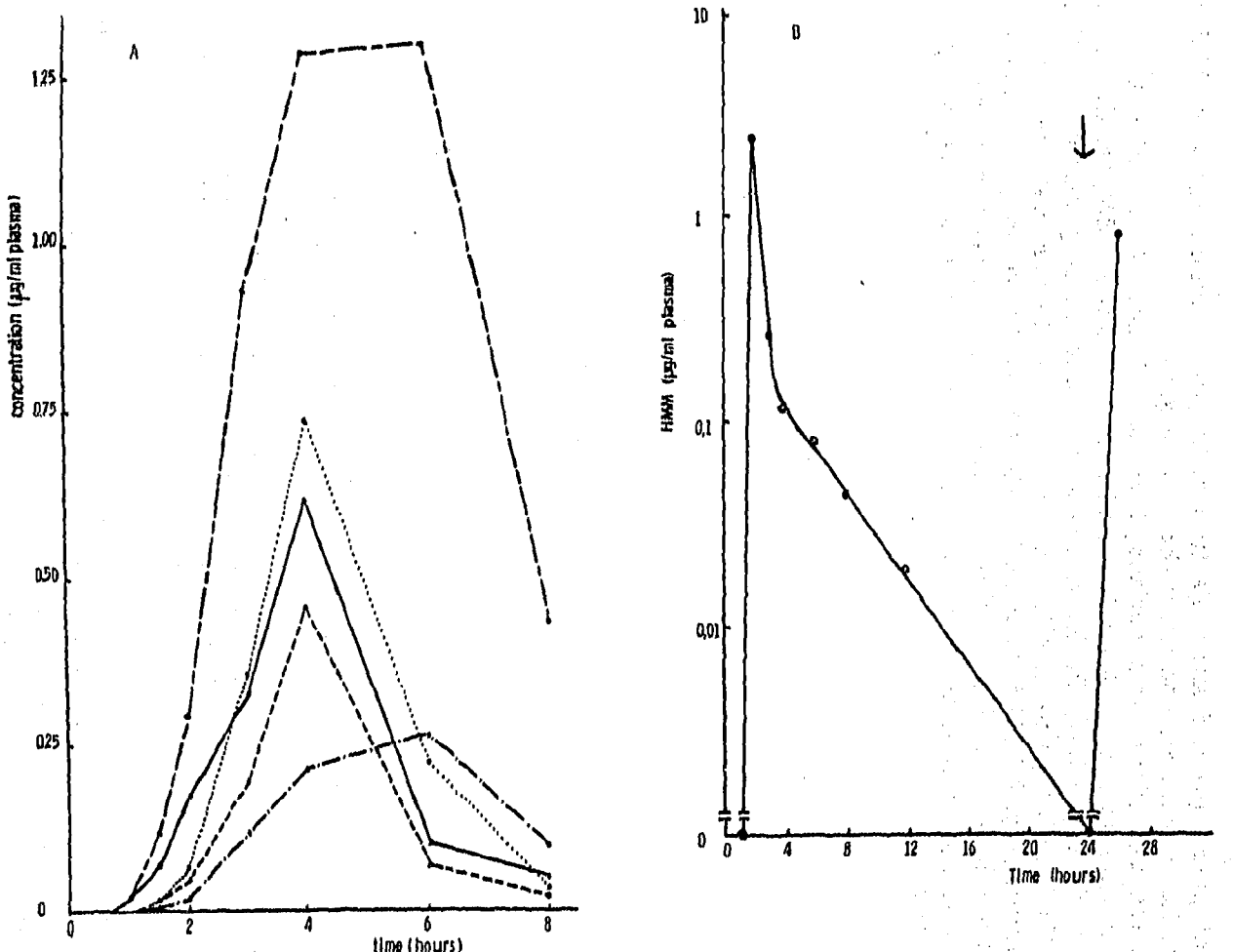


Fig. 3. (A) Plasma concentration—time curves for HMM (1), PMM (2), $TeMM_2$ (3), $TriMM_1$ (4), and $TriMM_2$ (5) from a patient after an oral dose of 300 mg of HMM. (B) Plasma concentration—time curve for HMM from the same patient after oral doses of 300 mg, given in a second medication sequence. The arrow denotes administration of second dose.

highest allowable temperature as mentioned by the manufacturer. We have used the same column daily for over three months without any perceptible loss of performance.

Except for the chromatographic column no glassware was silanized prior to use. Irregular recoveries of HMM, its metabolites and pethidine, due to adsorption onto the glass, were prevented by the addition of small volumes of an alcohol (methanol, isopropanol or amyl alcohol) during the various phases of the procedure.

Before extraction the pH of the plasma was adjusted to pH 9.5—9.7, which is more than four units above the reported pK_a value of HMM [17]. Mixing the serum samples with the extraction solvent by means of a vortex type mixer often resulted in the formation of emulsions, which could not be broken by centrifugation. No disturbing emulsification occurred if the tubes were shaken by hand. In view of the somewhat volatile nature of HMM, PMM, and particularly of the internal standard, pethidine, amyl alcohol was added to the organic extraction mixture before evaporation of the solvent; evaporation was stopped before the samples became entirely dry.

Calibration curves for HMM, PMM, $TeMM_1$, $TeMM_2$, $TriMM_1$ and $TriMM_2$ were constructed using the data obtained after the analysis of 10 pooled serum samples, spiked with HMM and its metabolites (5 different concentrations; 2 samples of each concentration). The calibration curves proved to be straight lines ($r^2 > 0.99$), passing through the origin. The curves could be extended to much higher concentrations of HMM and its metabolites than those obtained by adding to the samples 100 μl of the methanolic solution described under Materials and methods.

Five of the compounds which were investigated showed absolute recoveries of more than 80% (Table I). The absolute recovery of $TriMM_2$ was somewhat less, 74.4%, but still acceptable.

The reproducibility of the assay was examined by analyzing two series of six serum samples each, to which were added 10-μl and 100-μl portions of the methanolic solution (see Materials and methods) of HMM and metabolites, respectively. The results are shown in Table II.

TABLE II

PEAK-HEIGHT RATIOS (PHR) OF HMM AND METABOLITES TO PETHIDINE (INTERNAL STANDARD), AND COEFFICIENTS OF VARIATION (C.V.)

Results obtained with serum samples (1 ml) spiked with 10 μl or 100 μl of a methanolic solution of HMM and metabolites.

Compound	10 μl			100 μl		
	Concentration (ng/ml)	PHR*	C.V. (%)	Concentration (ng/ml)	PHR*	C.V. (%)
HMM	50.5	0.249	3.4	505	2.50	1.3
PMM	25.0	0.118	5.1	250	1.26	4.8
$TeMM_1$	9.7	0.040	5.0	97	0.409	3.2
$TeMM_2$	25.0	0.112	5.3	250	1.19	1.6
$TriMM_1$	10.8	0.038	7.9	108	0.327	4.3
$TriMM_2$	49.4	0.187	5.9	494	2.05	2.9

*Each value is the mean of 6 determinations.

The sensitivity of the method is such, that about 5 ng/ml plasma of each of the compounds could be quantitated with a coefficient of variation not exceeding 20% (results not shown). When analyzing plasma samples containing 5 ng/ml HMM, about 200 pg HMM is finally injected into the gas chromatograph. The detection limit for HMM under the prevailing chromatographic conditions was 30—40 pg HMM (signal-to-noise ratio, 5).

In order to examine the selectivity of the method, solutions of a number of compounds were injected and chromatographed following the same oven temperature program. These compounds were: metoclopramide, triethylperazine, prochlorperazine, 5-fluorouracil, glaphenine, triazolam, nitrazepam, flurazepam, doxorubicine, methotrexate, cyclophosphamide, cis-platinum, acetaminophen, acetophenetidin, caffeine, phenobarbital and bisacodyl. Of these compounds only acetophenetidin (retention time, 11.3 min) showed some interference with one of the methylmelamine peaks in the chromatogram.

The stability of the final amyl alcohol solutions obtained after extraction and evaporation was examined. Following chromatographic analysis some of the samples were stored for 24 h at room temperature, and then re-injected into the gas chromatograph. The peak-height ratios always remained constant within the experimental error, indicating that the amyl alcohol solutions are stable long enough to allow automation and the processing of large numbers of samples.

In Fig. 3A plasma concentration—time curves are shown for HMM, PMM, $TeMM_2$, $TriMM_1$ and $TriMM_2$, constructed from the analysis results of plasma samples of a 47 year old patient (55 kg) with normal renal function, who had received an oral dose of 300 mg HMM. After about 0.5 h, HMM and its metabolites appeared in the plasma samples. PMM, $TeMM_2$ and $TriMM_2$ levels were higher than the $TriMM_1$ and $TeMM_1$ levels, the latter compound being virtually absent. After having received no HMM for several weeks, a 14-day period started in which this patient were given daily doses of HMM. The plasma concentration of HMM, plotted on a logarithmic scale against time, after the first dose of 300 mg is shown in Fig. 3B. As reported before [8], the maximum plasma concentration was reached within a few hours, after which HMM disappeared from the plasma following a biphasic curve, indicating that drug absorption was fast as compared with drug distribution over the central and peripheral compartments. The halflife of the β-phase of elimination, determined graphically, was 2.9 h. HMM had practically disappeared from the plasma after 24 h, when the next dose was administered.

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