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RAPID, SENSITIVE DETERMINATION OF UNCHANGED PROMETHAZINE IN BIOLOGICAL MATERIAL USING A NITROGEN-SELECTIVE FLAME IONIZATION DETECTOR

IDENTIFICATION OF METABOLITES BY GAS CHROMATOGRAPHY–MASS SPECTROMETRY*

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SUMMARY

A rapid, sensitive method has been developed to study the kinetics of unchanged promethazine (PM) in biological material using a nitrogen-selective flame ionization detector (N-FID). Unchanged PM is distinguished from its desmethyl metabolite. Sample clean-up of several biological fluids (rat plasma, blood, urine, liver and kidney homogenates) was studied and gas chromatographic (GC) conditions optimized. Usually 50 μl–1.0 ml samples are extracted into *n*-heptane by shaking with NaOH, re-extracted into H_2SO_4 and again extracted into *n*-heptane by addition of NaOH. Finally, the organic phase is separated, concentrated under N_2 and PM determined by N-FID. However, a rapid, single-step method requiring only NaOH extraction into *n*-heptane may be used whenever GC background permits. Imipramine is used as an internal standard for calibration by peak height ratios in the overall range 5–1500 ng PM per sample. Recovery of both methods is high (97–99%) but precision of the single-step method is lower (relative S.D. 10% *versus* 3–4%). Use of sample volumes up to 1 ml allows accurate determination of concentrations as low as 10 ng/g. Examples of applications to commonly used animal models employing PM are given and simple adaptation for clinical samples suggested.

INTRODUCTION

Promethazine (PM, Fig. 1) is one of the original clinically used phenothiazine derivatives. It has found widespread clinical use as an anti-histamine and in the prevention of motion sickness. The anti-oxidant and surfactant properties of the compound have also led to its frequent use in chemistry, biochemistry and in animal

* These studies form part of the Ph.D. thesis of C.J.R.

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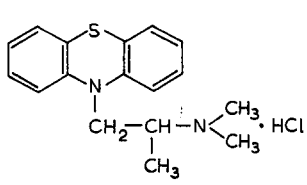
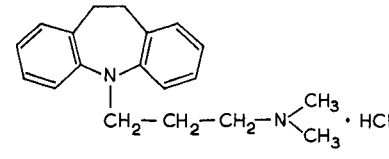


Promethazine hydrochloride
(N,N,α-Trimethyl-10H-phenothiazine-10-ethanamine)



Imipramine hydrochloride
(10,11-Dihydro-N,N-dimethyl-5H-dibenz
[b,f]azepine-5-propanamine)

Fig. 1. Structures of promethazine and internal standard imipramine.

studies of hepatotoxicity, especially those of the carbon tetrachloride model. Studies of the distribution kinetics of PM in laboratory animals have, however, not yet been reported. It was therefore of interest to develop a sensitive method for the determination of PM in biological material.

A recent review[1] of methods for determination of phenothiazine derivatives provided confirmation that gas chromatography (GC) is the present analytical method of choice. Several literature methods for GC determination of unchanged PM (or similar compounds) in biological material were therefore assessed[2–6] and nitrogen-selective flame ionization detection (N-FID) chosen as the system offering the best combination of specificity, sensitivity, accuracy and practicality. Although electron capture detection offers potentially lower detection limits, derivitization of the active substance to a halogen-containing compound is required. As PM-metabolites are not generally available, neither the specificity of the chosen derivatization reaction nor the possible reversion of PM-metabolites to parent compound during the reaction can be adequately monitored. Practical considerations therefore favour the use of non-derivatized samples.

MATERIALS AND METHODS

Reagents, glass-ware

All reagents used in the extraction process were of analytical grade. Organic solvents were tested for purity and possible GC interference by carrying out blank runs. Promethazine hydrochloride was a gift of Specia (Paris, France). Imipramine hydrochloride (Imip, Fig. 1) was provided by Ciba-Geigy (Basle, Switzerland). No special treatment of glass-ware is required.

Gas chromatography

The instrument used was a Perkin-Elmer Model 900 gas chromatograph equipped with a nitrogen-selective electrically heated rubidium silicate glass bead detector and linked to a Hewlett-Packard 3380 S integrator/recorder. Experience in this laboratory suggested the use of the polar Carbowax K 20M as a suitable liquid phase for separation of parent compound and possible metabolites. GC conditions were optimized for column length, temperature, carrier gas flow-rate and detector temperature. Biological samples from PM-treated rats were used in order to achieve maximum separation of PM and an adjacent metabolite peak. Final conditions adopted were: Column: Pyrex glass (1.8 m × 2 mm I.D.); liquid phase: 3% Carbo-

wax K 20M plus 1% KOH; support: Chromosorb W AW DMCS, 80–100 mesh; temperatures: injector 270°, oven 220°, detector 300°; flow-rates (ml/min): N_2 12, H_2 5, air 80; Bead potentiometer setting: 580.

Columns were conditioned as follows: without N_2 flow, heat slowly (1–2°/min) to 240°, hold for 1–2 h, cool and repeat; connect N_2 (flow-rate 17 ml/min) and run overnight (*ca.* 18 h); heat slowly to 240° again before adopting operating temperature.

Under these conditions, retention times for PM and an adjacent metabolite were *ca.* 5.8 and 6.2 min respectively. Imipramine was chosen as internal standard for reasons of availability and extremely practical retention time (*ca.* 3.6 min).

Standard solutions of PM and Imip (hydrochlorides) of 100 μg/ml in 0.01 *N* HCl were prepared for each analysis series and used for preparation of daily calibration control and internal standard solutions of 1.0 μg/ml.

Sample clean-up

The pH-dependence of extraction of PM into *n*-heptane was determined by use of an external–internal standard technique. Buffer solutions of varying pH were used in a first extraction step and the internal standard (Imip) added only during a second acidic back-extraction step. The resultant curves (Fig. 2) indicate that pH 9–13 is

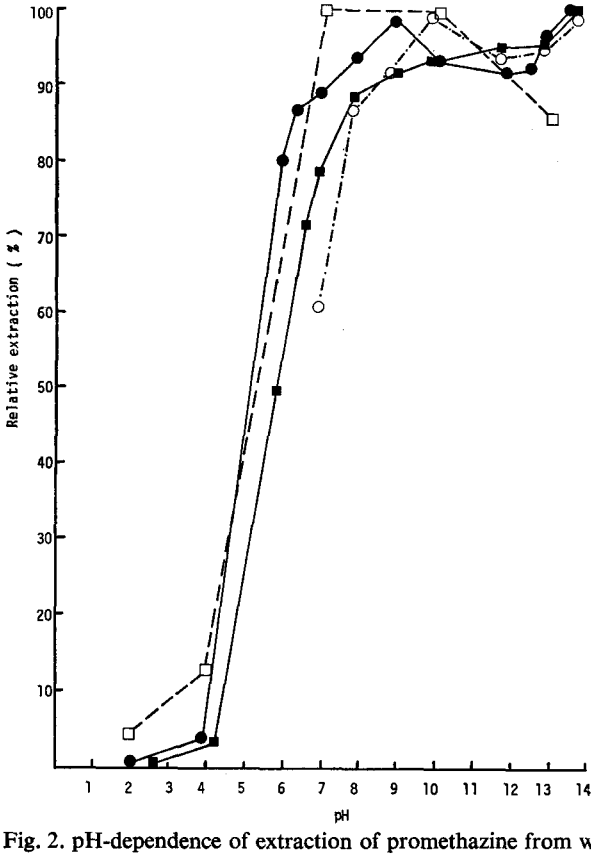


Fig. 2. pH-dependence of extraction of promethazine from water (●), liver homogenate (from PM-treated rats) (■), liver homogenate (spiked samples) (○) and blood (□) by *n*-heptane.

required for the efficient extraction of PM from all biological material tested. These relative results agree with the findings of similar absolute studies performed elsewhere using [^{14}C-]Imip (Fig. 3[7]). Further, constant peak height ratios (PM:Imip) are obtained at pH 9–11 when the two substances are extracted simultaneously, indicating that both behave similarly in this extraction procedure (Fig. 4).

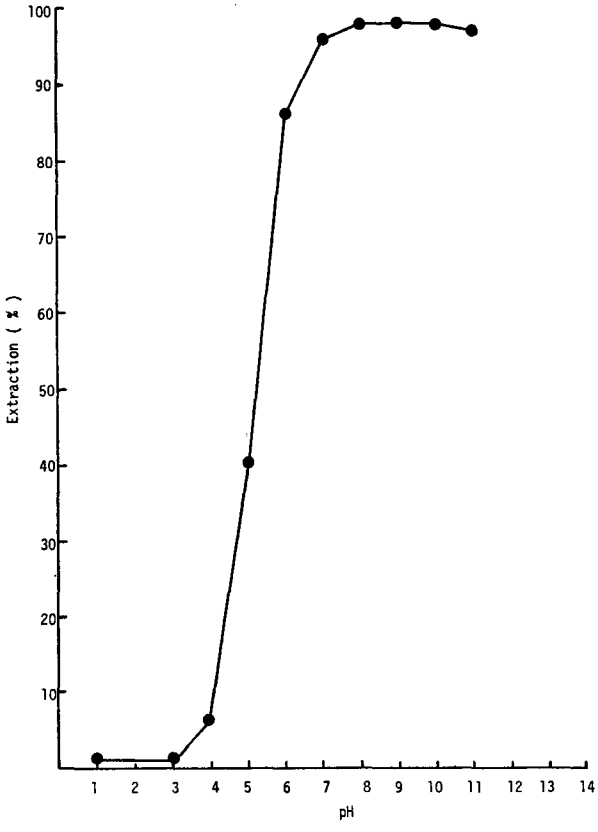


Fig. 3. pH-dependence of extraction of [^{14}C]imipramine from aqueous buffer solutions by *n*-heptane. Data from ref. 7.

Nevertheless, a detailed study of extraction of PM from each material was still required. For example, extraction from liver homogenate at pH 11.5 (2 *M* Na_2CO_3) was found to be dependent upon the dilution of the homogenate. Sodium hydroxide (1 *N*) was found to provide optimal extraction of the undiluted homogenate. Total lysis without clumping was found to be a pre-requisite for efficient extraction from whole blood and pH 10.0 (0.03 *M* borax buffer) optimal.

The effect of sonication (up to 50 kHz, 15 min) on extraction was evaluated for all biological material and found to be of little value to this method.

Extraction efficiency, as assessed by signal response, is unaffected by relatively large variations (50 μl–1.0 ml) in sample volume of the media tested (Fig. 5).

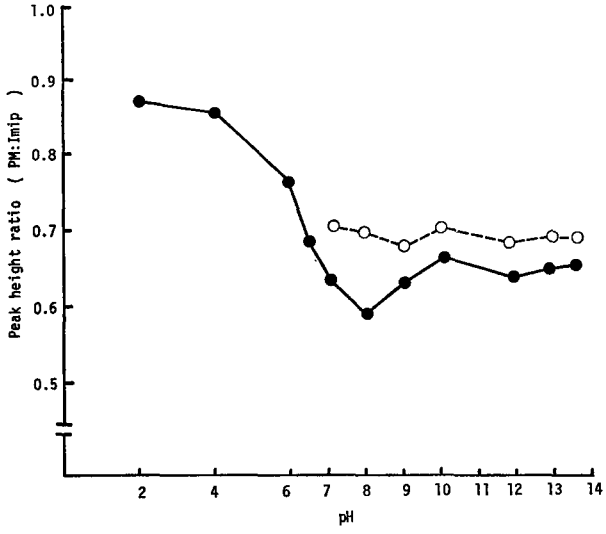


Fig. 4. Simultaneous extraction of promethazine and imipramine from water (●) and liver homogenate (○) under varying pH conditions by *n*-heptane.

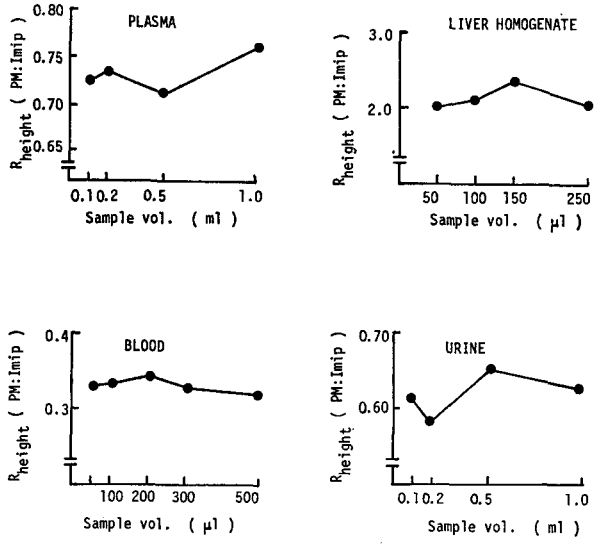


Fig. 5. Effect of sample volume on extraction efficiency as assessed by signal response and using an external–internal standard procedure.

Weighing of biological samples, especially homogenates and whole blood, afforded significant improvement in analytical precision and is recommended whenever micro-pipetting may be inaccurate due to the nature of the sample (*e.g.*, high viscosity).

Final procedures

50 μl (mg)–1.0 ml (g) plasma, blood, urine, liver or kidney homogenate
100–500 ng Imip (internal standard)
2 ml 0.03 *M* borax buffer, pH 10.0 (for blood) or 1 *N* NaOH (for others)
3 ml *n*-heptane–isopentyl alcohol (99:1)
Shake at 120 rpm for 10 min
↓
Centrifuge for 2–3 min at *ca.* 1000 *g*
↓
Remove maximum aliquot of organic phase to a fresh tube
Then *(A) Rapid method* (Recommended whenever GC background permits)
↓
Dry under N_2 at *ca.* 40° and re-dissolve residue in 50–200 μl *n*-heptane–isopentylalcohol (98:2) by spinning on a vortex mixer for 15 sec
↓
Inject 0.5–5.0 μl into the gas chromatograph
or *(B) Complete method* (For reduction of GC background)
↓
Shake with 2 ml 0.1 *N* H_2SO_4 at 120 rpm for 10 min
↓
Centrifuge and remove entire organic phase by pipette. Add 2 ml 1 *N* NaOH and shake with *n*-heptane–isopentyl alcohol (99:1) at 120 rpm for 10 min
↓
Centrifuge, remove maximum aliquot of organic phase to a fresh tube and complete as in (*A*), above.

Peak identification

Gas chromatographic–mass spectrometric (GC–MS) analysis of the individual chromatographic peaks obtained following extraction of biological samples was conducted with an Hitachi Perkin-Elmer RMU-6E instrument employing either the GC conditions described above or 3% OV-101 on Supelcoport (80–100 mesh) (all other conditions unchanged) and an ionizing potential of 70 eV at 180°.

RESULTS AND DISCUSSION

Calibration

A chromatogram typical for extracted samples of all biological materials tested shows that complete peak separation of PM and adjacent metabolite was not achieved under these conditions (Fig. 6). Comparative determinations using peak area and peak height ratios indicated that loss of the trailing tail area of the PM peak resulted in consistently lower content estimations. Hence, peak height ratios (PM : Imip) were used throughout for preparation of calibration graphs in the working ranges most appropriate for the material and within the overall range 5–1500 ng PM per sample (Fig. 7). Linear equations from regression analysis of calibration points were used for content determinations (Table I).

Recovery

In the absence of ^{14}C-labelled material, recovery was estimated by repeated extraction of the same sample. Multiple blood and urine samples from PM-treated rats were taken as examples and mean recoveries of 99% and 86% recorded. More-

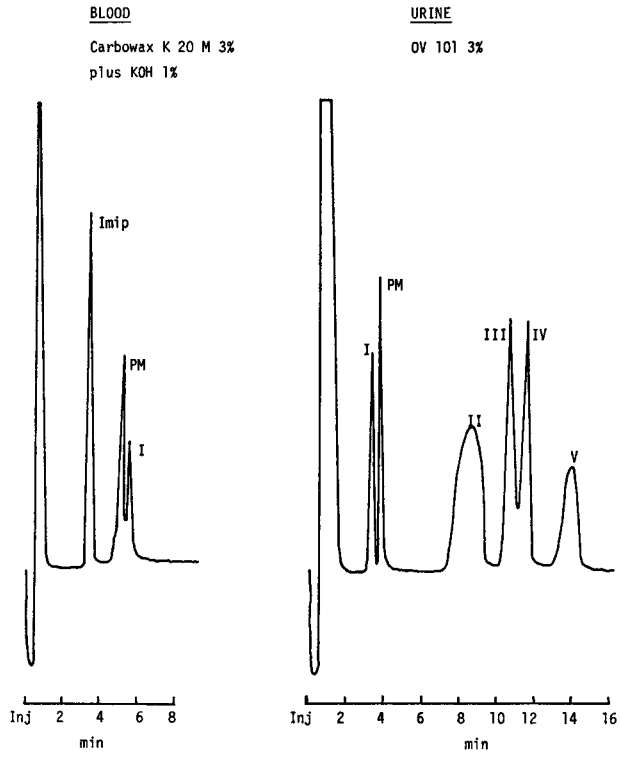


Fig. 6. Gas chromatograms recorded following extraction of blood and urine samples from PM-treated rats into *n*-heptane. For identification of compounds I–V, see Table III. For extraction procedures and full GC conditions, see text.

TABLE I

CALIBRATION GRAPH REGRESSION EQUATIONS USED FOR PROMETHAZINE SAMPLE CONTENT DETERMINATIONS

Biological fluid	*Internal standard (Imip) (ng per sample)*	*PM range (ng per sample)*	*Regression equation* $y = a \cdot x + b$*		*Correlation coefficient*	
			a	*b*	*r*	*(n)*
Water (=reference)	100	50–1500	0.00782	0.168	0.994	(20)
Liver homogenate	100	20–1500	0.00723	0.005	0.997	(40)
Blood	100	5– 100	0.00721	−0.009	0.999	(27)
Plasma	200	5– 40	0.00326	0.057	0.986	(45)
Kidney homogenate	200	5– 200	0.00311	0.012	0.996	(26)
Urine	500	100–1500	0.00140	−0.020	0.994	(25)

* y = Peak height ratio (PM:Imip); x = PM content (ng) of sample.

over, as the ratio sample weight: signal response was found to be linear for all biological material assayed, it may be assumed that recovery is consistent and substantial.

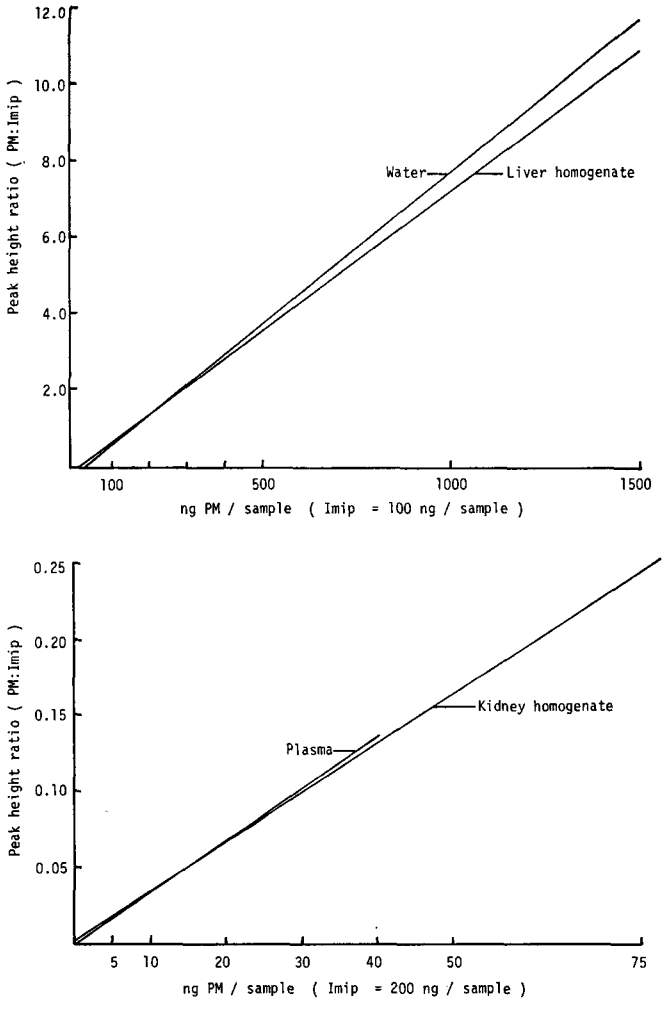


Fig. 7. Examples of calibration graphs. For details of linear regression analysis of these and other calibrations, see Table I.

Accuracy and precision

The accuracy of the extraction and calibration systems described above were established by analysis of samples containing amounts of PM within the expected range of biological samples, but unknown to the analyst. The results (Table II) indicate that the procedure is sufficiently accurate for determination of unknown biological samples: overall range of means, 97–99%.

Method precision was estimated by calculation of the relative standard deviation of repeated determination of the PM content of single biological samples from PM-treated rats (Table II). In the case of whole blood, precision was assessed by determination of five consecutive samples obtained by retro-orbital capillary sampling. Although a larger variance was calculated for whole blood (*ca.* 10% *versus ca.* 3% for other materials) this was considered to be satisfactory as the sampling method itself does not guarantee identical samples.

TABLE II

ACCURACY AND PRECISION OF DETERMINATION OF PROMETHAZINE IN BIOLOGICAL FLUIDS

Accuracy expressed as mean percentage recovery of n (in parentheses) different unknown samples ± relative standard deviation. Precision expressed as relative standard deviation of n (in parentheses) concentration determinations of the same biological sample from a PM-treated rat.

Biological fluid	*Accuracy (%)*	*Precision (%)*
Plasma	98.8 ± 8.6 (9)	3.4 (6)
Blood	99.1 ± 10.7 (6)	I 10.9 (5)
		II 7.4 (5)
Urine	98.8 ± 6.0 (6)	1.9 (5)
Liver homogenate	I 96.8 ± 2.4 (5)	4.0 (6)
	II 97.0 ± 3.0 (5)	
	III 98.2 ± 2.9 (5)	

Repeated GC injection of various extracted samples yielded an instrument variation averaging *ca.* 1%.

Sensitivity

Intensive study of the lower sections of the calibration graphs has not been undertaken as determination of samples containing less than 10 ng PM has been rarely necessary. Given the tested sample volume range, this value corresponds to a general sensitivity limit of 10 ng/g (ml) for the present method. However, the actual limit of sensitivity of the method is about 5 ng/g (ml).

Peak identification

GC–MS analysis of the individual chromatographic peaks was conducted using an extract from *ca.* 10 ml of urine from PM-treated rats (Fig. 6). Identification of apparent PM and the five metabolites was made from the principal mass fragments recorded (Table III).

Key fragments at m/e values 58 and 30 are considered to point to the unambiguous N-demethylation (as distinct from C-demethylation) of the PM side-chain in the desmethyl metabolite (I). Identification of the sulphoxide (IV) was confirmed by GC retention time and MS analysis of the oxidation product of sodium periodate-treated PM: m/e value 300 ($=M^+$). Additionally, the oxidation product could not be silylated, evidence that the addition is not phenolic. The proposed dioxide metabolite (V = prothanon) was similarly confirmed by GC retention time analysis of the oxidation product of potassium permanganate-treated PM. However, the broader peaks of metabolites II and V may be indicative of the presence of small amounts of other metabolites not resolved by the GC system employed. The structure assignments of these compounds should therefore be regarded as tentative.

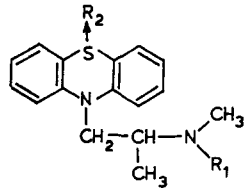
Stability

Stock solutions of PM and Imip (100 μg/ml in 0.01 *N* HCl) were found to be stable for up to 50 days when refrigerated (4°, dark, stoppered flasks). As a precautionary measure, however, these solutions were only used for preparation of dilute standard solutions (1 μg/ml) for 7 days after manufacture[8].

TABLE III

PEAK IDENTIFICATION BY GC–MS

Principal mass fragments recorded for apparent PM and metabolites I–V (see Fig. 6). For GC–MS conditions, see the text.



Compound	*Proposed structure*		*Key fragments (m/e values)**
	R_1	R_2	
PM	CH_3	—	M^+ = 284 (44), 213 (27), 212 (0.9), 199 (3), 198 (41), 180 (44), 72 (100), 44 (50)
I	H	—	M^+ = 270 (7), 213 (84), 198 (25), 180 (24), 72 (17), 58 (100), 30 (9)
II	H	O	M^+ = 286: poor definition
III	H	O_2	M^+ = 302 (2.8), 245 (8), 244 (5.7), 58 (100)
IV	CH_3	O	M^+ = 300 (0.5), 284 (1.5), 213 (2.6), 212 (1.5), 199 (3.2), 198 (4), 180 (1.8), 72 (100), 58 (19)
V	CH_3	O_2	M^+ = 316 (2.5), 72 (100): poor definition

* Relative abundances are given in parentheses.

Samples of *n*-heptane containing PM and Imip ready for GC injection were found to be stable for 24 h at room temperature (23°, light/dark) and up to 72 h at −20° (deep-freeze). Samples should be of minimum volume (less than 200 μl) and be stored in tightly sealed glass tubes.

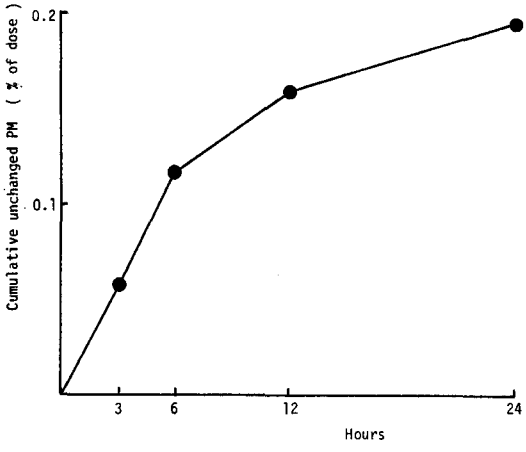


Fig. 8. Cumulative elimination of unchanged PM in urine following a single i.p. dose of 25 mg/kg to a fasted, 200 g, male Tif: RAI f (SPF) rat.

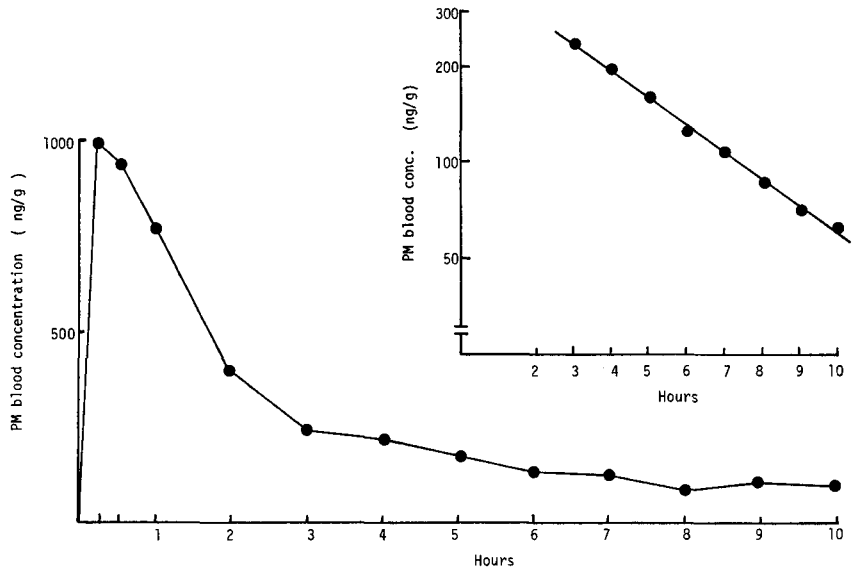


Fig. 9. Blood concentrations of unchanged PM following a single i.p. dose of 25 mg/kg to a fasted, 200 g, male Tif: RAI f(SPF) rat.

Application

In order to test the general application of the method, the urinary elimination and blood concentration: time course of unchanged PM were monitored in a single rat following a single intra-peritoneal dose of 25 mg/kg. Blood was collected using a retro-orbital capillary serial sampling technique.

Unchanged PM was found to be eliminated renally in only very small quantities (Fig. 8), *ca.* 0.2% of the administered dose appearing in the first 24 h. Plotting the blood concentration data (Fig. 9) on a semi-logarithmic scale permits estimation of the biological half-life of the drug. For unchanged PM this appears to be about 3.5 h.

It is concluded that the method is satisfactory for determination of the distribution kinetics of unchanged PM. As neither human plasma, whole blood nor urine (in sample volumes of up to 1 ml) give rise to interfering GC background, the method may easily be adapted to clinical samples by establishing the optimum extraction pH and preparing calibration graphs. Should small quantities be available for extraction comparability and calibration, it may also be possible to extend the method to simultaneous determination of PM-metabolites I–V (Table III).

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REFERENCES

1 J. Blazek, A. Dymes and Z. Stejskal, *Pharmazie*, 31 (1976) 681.
2 M. Riedmann, *Xenobiotica*, 3 (1976) 411.
3 M. Riedmann, *J. Chromatogr.*, 92 (1974) 55.
4 C. M. Davis, C. J. Meyer and D. C. Fenimore, *Clin. Chim. Acta*, 78 (1977) 71.
5 H. B. Hucker and S. C. Stauffer, *J. Chromatogr.*, 138 (1977) 437.
6 F. A. J. vanderHeeren, D. J. C. J. Theunis and M.-T. Rosseel, *J. Chromatogr.*, 120 (1976) 123.
7 W. Riess, L. Dubey and J. Godbillon, Ciba-Geigy Ltd., Basle, unpublished results.
8 B. J. Meakin, J. Stevens and D. J. G. Davies, *J. Pharm. Pharmacol.*, 30 (1978) 75.