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DETERMINATION OF N,N-DIMETHYLDIBENZO[*b,f*]THIEPIN-10-METHYLAMINE BY GAS–LIQUID CHROMATOGRAPHY

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SUMMARY

A specific and sensitive gas chromatographic method has been developed for the determination of unmetabolized N,N-dimethyldibenzo[*b,f*]thiepin-10-methylamine in biological fluids. The dosage of this hypnotic compound is relatively low (25–50 mg). The average maximum concentration in blood lies between 30 and 40 ng/ml (after a 40-mg oral dose). The high sensitivity required was achieved by reacting DTM with ethyl chlorocarbonate to yield ethyl N,N-dimethylcarbamate and the corresponding chloromethylene derivative. The latter could then be detected with an electron capture detector.

INTRODUCTION

N,N-Dimethyldibenzo[*b,f*]thiepin-10-methylamine (DTM) is a hypnotic substance when administered to animals and man. In order to investigate its pharmacokinetic properties, a sensitive and specific method was needed in order to assay the unchanged drug in biological fluids. Gas chromatography (GC) using a flame ionization detector was found to be sufficiently sensitive for measuring the elimination of DTM in urine, but not sensitive enough for determining the low concentrations that arise from therapeutic doses in blood and plasma. We found that a GC method described by Schöne and co-workers[1,2], using a nitrogen-specific flame detector, produced too low a sensitivity and an unacceptable variation in the results.

The gas–liquid chromatographic (GLC) method described in this paper takes advantage of the unique reaction of DTM with ethyl chlorocarbonate to yield ethyl N,N-dimethylcarbamate and dibenzo[*b,f*]thiepin-10-methylene chloride. The latter is a neutral product and is electron-capturing, and can be detected with high sensitivity. A single determination requires only 1 ml of blood. The accuracy achieved by this method is attained by adding an internal standard directly to the biological sample prior to the extraction process. This internal standard differs from DTM only in carrying a chlorine substituent on one of the aromatic rings. It displays the same extraction properties as DTM and undergoes the same chlorocarbonic acid ester degradation.

The N-desmethyl derivative of DTM, which may be expected as a potential

metabolite, undergoes a different reaction with chlorocarbonic acid ester to yield ethyl N-(dibenzo[*b*,*f*]thiepin-10-methylene)-N-methylcarbamate.

This specificity of reactions led to the selective, quantitative assay of unchanged DTM in biological fluids.

OPTIMIZATION OF ANALYTICAL CONDITIONS

The results described below were obtained by using ^{14}C-labelled DTM with a specific radioactivity of 15.5 μCi/mg.

Extraction

Most organic solvents extract DTM from aqueous phases under alkaline conditions. Hexane was chosen as the solvent in this work because of its relatively low volatility and because it can be purified easily. The pH dependence of the extractability of DTM as shown in Fig. 1 shows that at a pH of the aqueous phase above 7.0, the labelled DTM is completely extracted. DTM can be back-extracted from the organic phase into fresh aqueous phase at pH $\leqslant$ 1 and re-extracted from there at pH > 7.0.

Using a sample volume of up to 3 ml, neither urine, plasma nor whole blood contains significant impurities that are co-extractable and interfere with the GLC peaks of the derivatives of DTM and the internal standard.

The reaction schemes for the formation of DTM derivatives are shown in Fig. 2.

DTM (I) and the internal standard (IV) react quantitatively with ethyl chlorocarbonate to yield ethyl N,N-dimethylcarbamate (III) and the respective chloromethylene derivatives (II and V), which can be sensitively assayed by means of an electron capture detector. In contrast, the desmethyl derivative of DTM (VI) reacts under the same conditions to yield ethyl N-(dibenzo[*b*,*f*]thiepin-10-methylene)-N-methylcarbamate (VII) and hydrogen chloride.

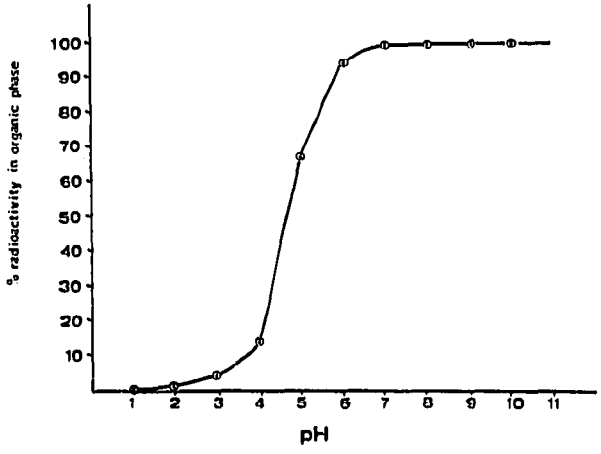

Fig. 1. pH dependence of the partition between buffer (pH 1–11) and heptane.

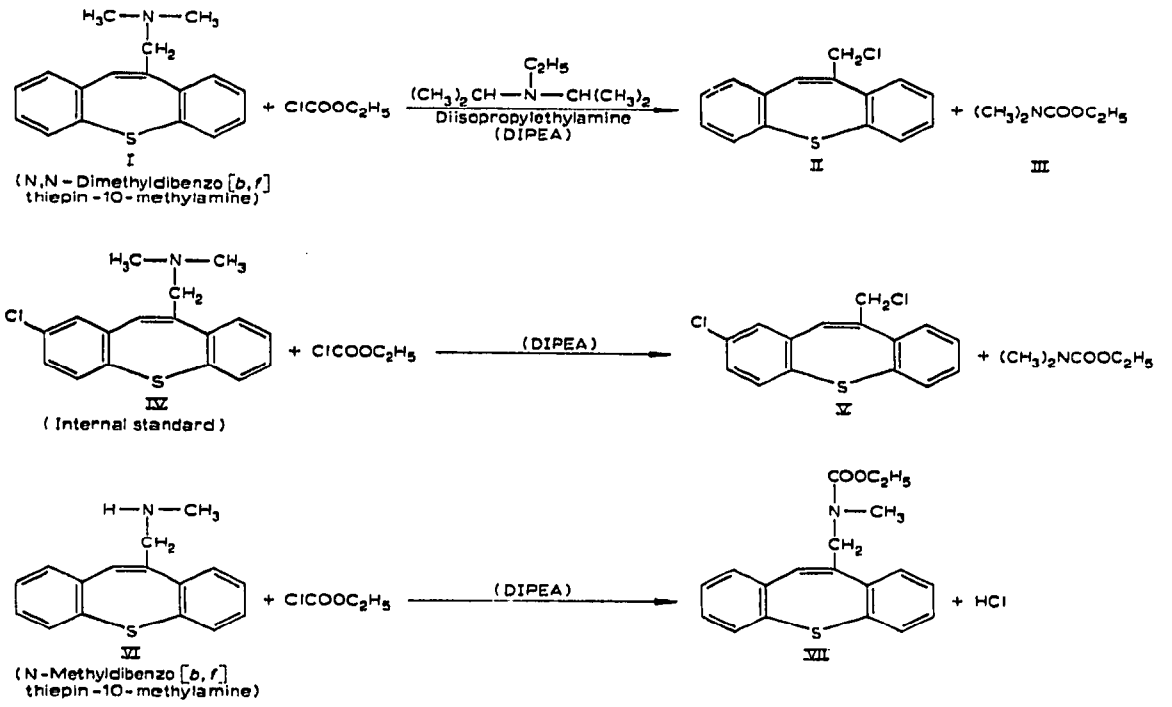

Fig. 2. Reaction schemes for the formation of DTM derivatives.

As ethyl chlorocarbonate always contains trace amounts of hydrochloric acid, which blocks the formation of the quaternary ammonium intermediates, the addition of diisopropylethylamine, which itself cannot be quaternized, was found to be necessary in order to achieve quantitative yields of the end products.

As shown in Fig. 3, the reaction is complete after 30 min at 60°.

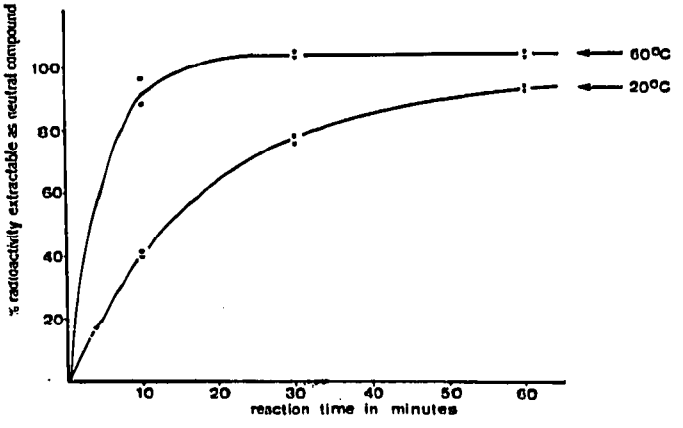

Fig. 3. Rate of reaction of ethyl chlorocarbonate and N,N-dimethyldibenzo[*b*,*f*]thiepin-10-methylamine. The values exceed the 100% limit owing to the slight evaporation of hexane during the manipulations.

In order to ascertain the structures of the end products, the free bases of both DTM and the internal standard, as well as the desmethyl derivative, were subjected to the above reaction in milligram amounts, and worked up for neutral material. The gas chromatograms of the three products showed single peaks and the mass spectra were in accordance with the structures II, V and VII.

Gas chromatography

The instruments used were a Pye Unicam Model 74, Series 104, gas chromatograph with a pulsed 150 μsec electron capture detector (^{63}Ni, 10 mCi), an Infotronics Model CRS 208 integrator and a W+W Model 1100 recorder. The columns were 3 ft. long and made of Pyrex glass, containing 1% SE-30 on Chromosorb G, acid-washed and dimethylchlorosilylated, 80–100 mesh. The temperature of the column oven was 180°, of the detector 350° and of the injector 200°. The carrier gas was nitrogen at a flow-rate of 50 ml/min.

The columns were conditioned as follows: (1) for 2 h at 250° without a flow of nitrogen, and (2) for 24 h at 280° with nitrogen at a flow-rate of 10 ml/min.

The retention times of the derivatives of DTM and internal standard are about 4 and 7.2 min, respectively, using the described conditions.

The detector response was evaluated by injecting mixtures containing a fixed amount of derivatized internal standard (V) and various amounts of the DTM derivative. The peak area ratios were plotted against the amount of DTM in nanograms per 100 μl of sample (Fig. 4). This response graph proved to be a linear function between 2.5 and 200 ng of DTM (using 26.5 ng of internal standard per sample). It should be noted that the amount injected must not exceed *ca.* 2 ng for either compound. Therefore, it is necessary for samples that contain more than 50 ng per 100 μl to be diluted with hexane. If all samples up to 200 ng would be dissolved in 100 μl of hexane, the response graph would cease to be linear above *ca.* 100 ng.

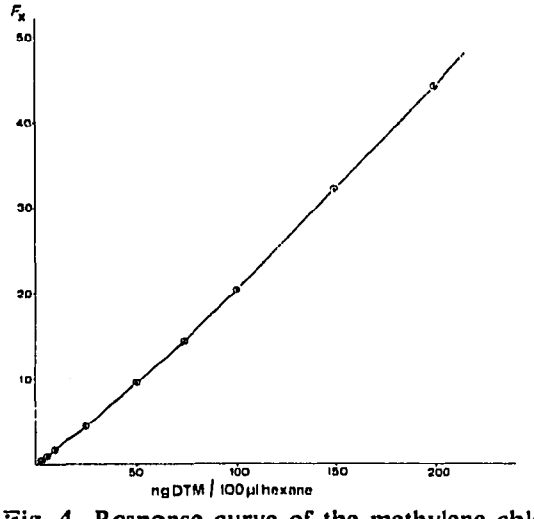

Fig. 4. Response curve of the methylene chloride derivative (II) of DTM. F_x= peak area ratio of DTM to internal standard. Internal standard= 26.5 ng of the methylene chloride derivative (V) per 100 μl of hexane. Injected volume= 5 μl (of 100 μl).

The stability of the GC system was checked over a period of 2 months by daily injections of two or three samples containing various amounts of DTM derivative (II) and 26.5 ng of internal standard derivative (V).

It was found that the deviation from the original response graph never exceeded 10%. The standard deviation of a 50-ng sample (50 ng of DTM derivative plus 26.5 ng of internal standard per 100 μl of hexane) over the 2-month period was $\pm$4.5%.

MATERIALS AND METHODS

Reagents

All of the chemicals used were of analytical grade and were specially tested for purity by carrying out blank runs.

Extraction procedure and derivative formation

The procedure was carried out according to the following scheme:

1–3 ml of serum, urine or whole blood; 1.0 ml of internal standard (IV) (28.5 ng per millilitre of water); 2.0 ml of buffer (pH 10.0);
5.0 ml of hexane containing 1% of isoamyl alcohol.
The mixture is agitated for 10 min using a mechanical shaker at *ca.* 170 rpm.

↓

Centrifuged for 5 min at *ca.* 3000 rpm (rotor radius = 7 cm).

↓

A maximum aliquot of the hexane phase is removed and shaken with 2 ml of 0.1 *N* H_2SO_4 for 10 min at *ca.* 170 rpm.

↓

The hexane phase is separated by aspiration; 1 ml of 1 *N* NaOH and 5 ml of hexane are added to the remaining aqueous phase and the mixture is shaken for 10 min at *ca.* 170 rpm.

↓

A maximum aliquot of the hexane phase is removed and dried with a stream of N_2 at *ca.* 50°.

↓

The dry residue is dissolved in 1.0 ml of hexane; 20 μl of diisopropylethylamine and 10 μl of ethyl chlorocarbonate are added and the mixture is heated for 1 h at 60° in a water-bath. Then 2.0 ml of 0.1 *N* H_2SO_4 and 4.0 ml of hexane are added to the cooled reaction mixture and shaken for 5 min at *ca.* 250 rpm.

↓

A maximum aliquot of the hexane phase is removed and shaken with 2.0 ml of 0.1 *N* NaOH for 5 min at *ca.* 250 rpm.

↓

A maximum aliquot of the hexane phase is removed and evaporated to dryness with a stream of N_2 at *ca.* 50°.

↓

The dry residue is dissolved in at least 100 μl of hexane.

↓

The sample is now ready for GLC.

Calibration graphs from biological material

Calibration graphs from whole blood, plasma and urine were prepared by adding both DTM and internal standard to a fresh biological specimen and following the complete analytical procedure. It was found that there was no significant difference between the extractabilities from the various biological fluids.

For the clinical experiments, two calibration graphs for whole blood were prepared, one covering the range 3.8–242 ng of DTM per millilitre of blood (using 28.5 ng/ml of internal standard) and the other covering the low concentration range from 2.4 to 12 ng per millilitre of blood (using 5.70 ng/ml of internal standard). The graphs are shown in Figs. 5 and 6.

The increased accuracy of the second calibration graph is due to the smaller amount of internal standard added to the sample, which produces a more favourable peak area ratio. The same calibration curves are also valid for plasma, serum, urine and water.

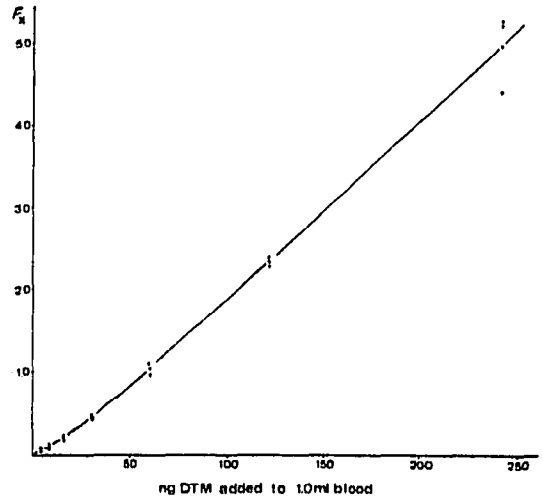

Fig. 5. Calibration graph for the whole analytical procedure at high concentrations. F_x = peak area ratio of DTM to internal standard (28.5 ng per millilitre of blood). Injected volume= 5 μl (of 100 μl).

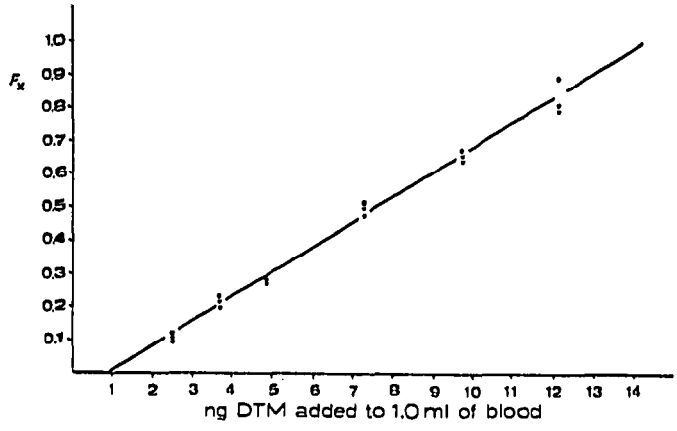

Fig. 6. Calibration graph for the whole analytical procedure at low concentrations. F_x= peak area ratio of DTM to internal standard. (5.70 ng per mililitre of blood). Injected volume= 5 μl (of 100 μl).

Analyses of test samples

The calibration graphs as well as the entire procedure were tested by analysing prepared samples with fixed concentrations that were unknown to the analyst. Twelve such samples of whole blood were analyzed. Three extractions on 1 ml of blood were carried out for each sample, and 28.5 ng of internal standard (in 1 ml of water) was added to each portion prior to extraction.

RESULTS

The results are given in Table I.

TABLE I

RESULTS OF THE ANALYSIS OF 12 SAMPLES OF WHOLE BLOOD FOR DTM

Sample No.	*Actual concentration (ng/ml)*	*Found concentration (ng/ml)*	*Deviation (%)*
1	111.5	105	− 5.8
2	15.1	14.0	− 7.3
3	198.0	172.0	−13.1
4	5.0	5.15	+ 3.0
5	194.0	210	+ 8.0
6	5.3	5.0	− 5.7
7	242.0	258	+ 6.6
8	0	0	0
9	130.3	130.0	− 0.2
10	15.1	12.1	−19.3
11	96.8	96.5	− 0.3
12	26.9	33.7	+ 25

Comparison of the GLC method with the radioisotope dilution technique

Objective. In order to test the GLC method in the presence of naturally occurring metabolites of DTM, blood and urine samples obtained from a healthy human volunteer following the ingestion of 40 mg of ^{14}C-labelled DTM were to be analyzed by this method and by the conventional technique of radioisotope dilution analysis.

Procedure. The volunteer received a single dose of 40.0 mg of ^{14}C-labelled DTM (100 μCi) orally in a gelatine capsule. In order to obtain sufficiently large

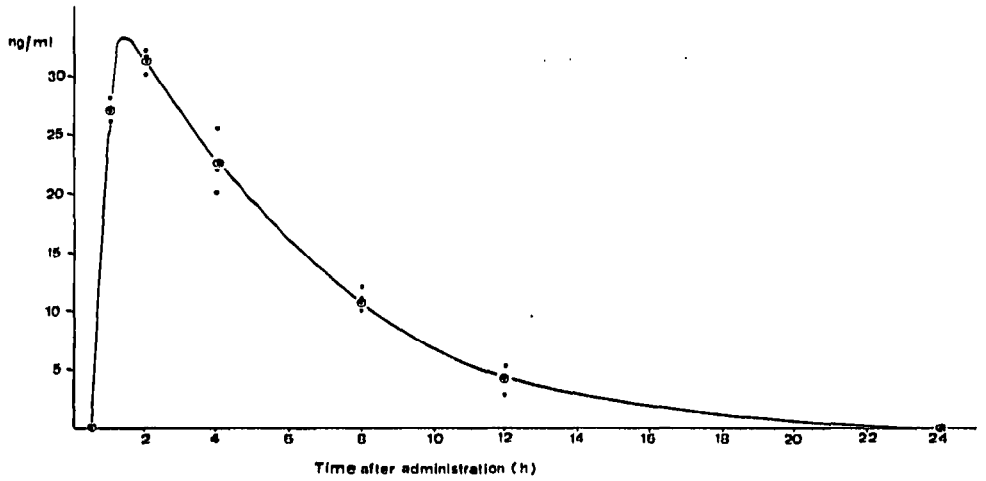

Fig. 7. Concentrations of unchanged drug in the blood following a single, oral dose of 40 mg of DTM to subject G. D. (71 kg).

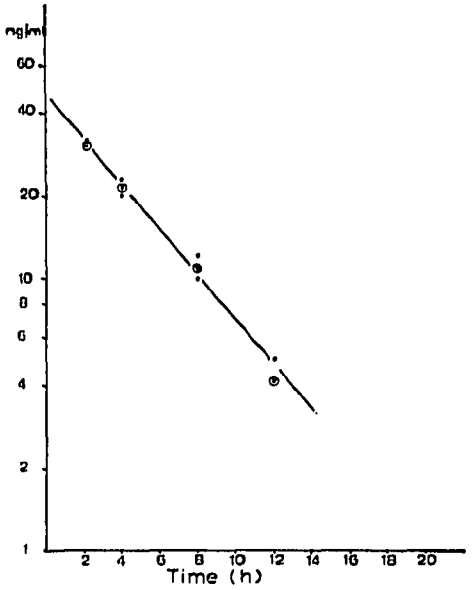

Fig. 8. Decline of unchanged drug (ng/ml) in the blood following a single, oral dose of 40 mg of DTM to subject G. D. (71 kg). Biological half-life (t_{50}) = *ca.* 4 h.

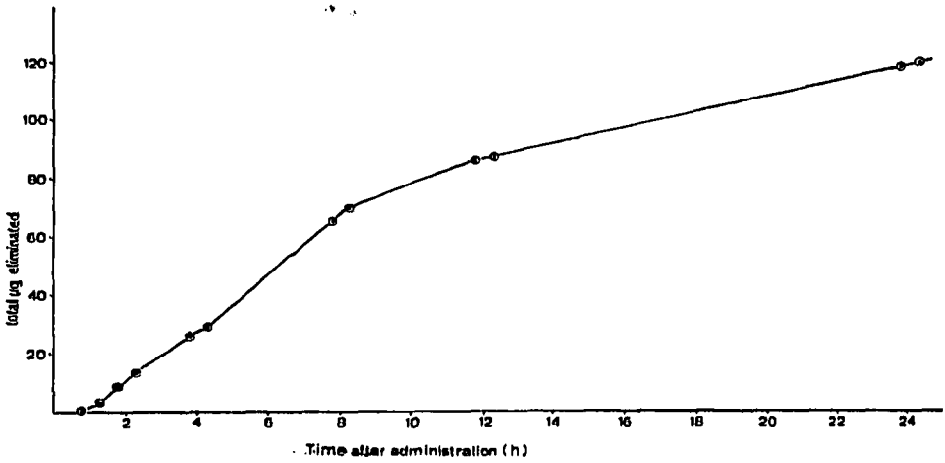

Fig. 9. Cumulative elimination of unchanged drug in the urine following a single, oral dose of 40 mg of DTM to subject G. D. (71 kg).

TABLE II

CONCENTRATION OF UNCHANGED DRUG IN URINE AFTER A SINGLE, ORAL DOSE OF 40 mg OF ^{14}C-LABELLED DTM TO A HUMAN SUBJECT (G. D., MALE, 71 kg)

Collection interval time (h)	*Volume of urine (ml)*	*pH of urine*	*DTM by GLC (ng/ml)*	*DTM by IDA* (ng/ml)*	*Cumulative total elimination (µg)*	*Rate of elimination (µg/h)*
$-\frac{1}{2}$–0	24	5.1	0	—	0	0
0–$\frac{3}{4}$	37	5.25	5.3	—	0.196	0.196
$\frac{3}{4}$–$1\frac{1}{4}$	115	6.45	24.0	—	2.956	5.52
$1\frac{1}{4}$–$1\frac{3}{4}$	335	6.80	17.5	—	8.818	11.73
$1\frac{3}{4}$–$2\frac{1}{4}$	110	5.85	45.7	—	13.845	10.05
$2\frac{1}{4}$–$3\frac{3}{4}$	200	6.15	63.2	—	26.490	8.43
$3\frac{3}{4}$–$4\frac{1}{4}$	78	6.65	39.5	44.0	29.570	6.16
$4\frac{1}{4}$–$7\frac{3}{4}$	115	5.20	315	—	65.710	10.35
$7\frac{3}{4}$–$8\frac{1}{4}$	15	5.20	265	260	69.770	7.95
$8\frac{1}{4}$–$11\frac{3}{4}$	815	5.85	21.0	—	86.880	4.89
$11\frac{3}{4}$–$12\frac{1}{4}$	43	6.0	19.75	19.0	87.730	1.699
$12\frac{3}{4}$–$23\frac{3}{4}$	395	5.20	78.5	82.0	118.740	2.70
$23\frac{3}{4}$–$24\frac{1}{4}$	6	5.10	145	—	119.610	1.74

Time (h)	*Percentage of dose in urine*	
	Unchanged DTM	*Total radioactivity*
0–24	0.3	53.8

* Inverse radioisotope dilution analysis.

urine samples at the requested intervals, the volunteer received 200 ml of drinking water per hour. Blood samples were drawn at 1/2, 1, 2, 4, 8, 12 and 24 h after administration of the dose and urine samples were collected at intervals covering the total urinary elimination from 0 to 24 h after administration.

All samples were analyzed for unchanged DTM by the GLC method and selected samples were independently analyzed by the radioisotope dilution technique. The heparinized blood samples and the urine samples were kept frozen until the analysis was carried out.

Results. The highest blood concentration appeared in the 2-h sample (Fig. 7), but the maximum concentration is probably reached before that time because the interval from $1^1/_4$ to $1^3/_4$ h shows the highest urinary elimination rate. For the determination of a biological half-life, the declining part of the blood concentration curve was plotted on semi-logarithmic graph paper. The biological half-life appears to be about 4 h (see Fig. 8). The total amount of unchanged DTM excreted in urine from 0 to 24 h represents 0.3% of the dose administered (Fig. 9, Table II).

The GLC and radioisotope dilution methods gave acceptable agreement

TABLE III

CONCENTRATIONS OF UNCHANGED DRUG IN WHOLE BLOOD AFTER A SINGLE, ORAL DOSE OF 40 mg ^{14}C-LABELLED DTM TO A HUMAN SUBJECT (G.D., MALE, 71 kg) AS DETERMINED BY GLC ANALYSIS AND RADIOISOTOPE DILUTION ANALYSIS AND TOTAL RADIOACTIVITY EXPRESSED IN TERMS OF DTM (ng/ml) REPRESENTING THE SUM TOTAL OF METABOLITES PLUS UNCHANGED DTM

Time after application (h)	*Unchanged DTM by GLC analysis*		*Unchanged DTM by inverse radioisotope dilution analysis (ng/ml)*	*Total radioactivity (ng/ml)*
	Single values (ng/ml)	*Average values (ng/ml)*		
1/2	< 1.0 < 1.0 < 1.0	< 1.0	–	15
1	26.0 27.0 28.0	27.0	34.0	117
2	30.0 31.5 32.0	31.2	–	171
4	20.0 25.5 22.0	22.5	25.0	162
8	10.0 10.0 12.0	10.7	13.0	131
12	5.2 2.7 4.2	4.0	8.0	109
24	< 1.0 < 1.0 < 1.0	< 1.0		75

between the results for the urinary samples (Tables II and III). With blood, the isotope dilution technique invariably produced higher values than GLC analysis. However, when 200 mg of carrier was added to 10 ml of blood, then re-isolated and re-crystallized, specific radioactivities in the range 3–10 dpm/mg only were obtained. No guarantee can be given that the limited number of crystallizations removed all of the metabolites.

As all of the values obtained from the radioisotope dilution procedure represent single determinations, no measure of deviation can be given.

Fig. 10 shows a typical gas chromatogram of a blood sample taken 2 h after administration of 40 mg of ^{14}C-labelled DTM, and a chromatogram of a blank, containing internal standard only.

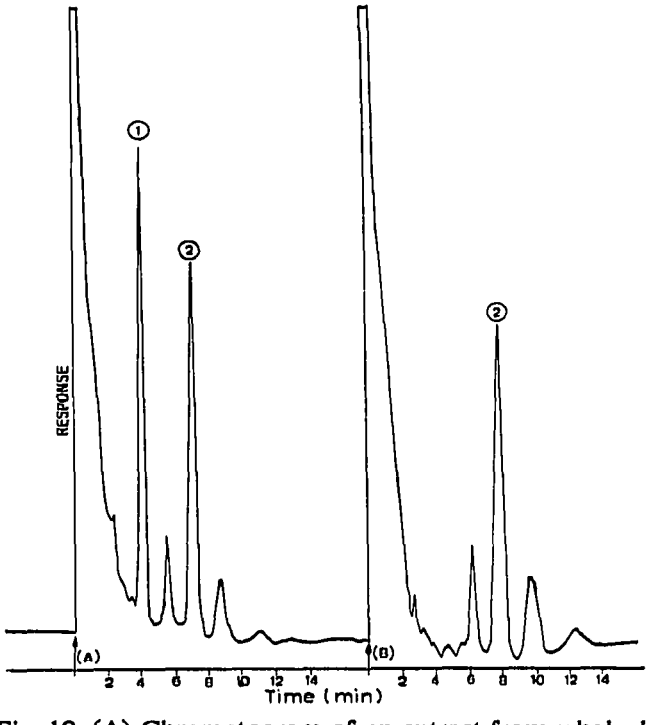

Fig. 10. (A) Chromatogram of an extract from whole, human blood containing 35.0 ng of DTM per millilitre of blood (sample taken 2 h after administration of 40 mg). (B) Chromatogram of an extract from whole, human blood containing internal standard only (28.5 ng/ml). Both extracts were derivatized and finally dissolved in 100 μl of hexane and 5 μl of each was injected. Attenuation $\times$128; range $\times$10. 1 = DTM derivative (II); 2 = internal standard derivative (V).

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