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DETERMINATION OF THERAPEUTIC LEVELS OF METRONIDAZOLE IN PLASMA BY GAS–LIQUID CHROMATOGRAPHY

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SUMMARY

A procedure is described for the quantitative determination of metronidazole (1-β-hydroxyethyl-2-methyl-5-nitroimidazole) in human plasma. After extraction from plasma, metronidazole and the internal standard, 1-β-hydroxyethyl-2-methoxy-5-nitroimidazole, are converted to trimethylsilyl derivatives for injection into a gas chromatograph. Detection by means of a flame ionization detector allowed concentrations of 0.25 μg of metronidazole per ml plasma to be determined with a precision of 6%. Application of the assay to obtain profiles of metronidazole in plasma of dog and man after single doses of 12 mg/kg and 250 mg, respectively, are reported.

INTRODUCTION

Metronidazole (I) is the drug of choice in the treatment of *Trichomonas* infections and is also a very effective amebicide. Since it has been suggested that certain individuals who responded poorly to metronidazole also exhibited low plasma levels, either from ineffective absorption (Kane *et al.*[1]) or because of rapid metabolism (Stambaugh *et al.*[2]), there has been a need for sensitive specific procedures of assay of this drug.

The classical method of assay of I described by Kane *et al.*[1], which was refined by Taylor *et al.*[3], depends on a polarographic analysis that detects all drug-related material containing the intact nitroimidazole nucleus and thus lacks specificity. The more recent method of Welling and Monro[4] depends upon thin-layer chromatographic (TLC) separation and quantitation by densitometry and, while specific and sensitive, appears cumbersome for routine analysis.

The absorptiometric assay of Lau *et al.*[5] is based on the alkaline hydrolysis of N-1-substituted 5-nitroimidazoles to yield stoichiometric amounts of the nitrite ion. Acidification is used subsequently to diazotize sulfanilamide, which is coupled with N-(1-naphthyl)ethylenediamine to produce the characteristic Bratton–Marshall azo dye chromophore. This procedure lacks specificity as the azo compounds of N-1-substituted 2-nitroimidazoles and metabolites also give this color.

The methods described by De Silva *et al.*[6] for estimating N-substituted nitro-

imidazoles in blood and urine required a TLC step to separate out unchanged drug before quantitation by an absorptiometric, polarographic, or gas chromatographic procedure. In this paper we report an assay of metronidazole from plasma by gas–liquid chromatography (GLC) employing a flame ionization detector, which avoids TLC cleanup and is applicable to routine estimation of plasma levels following single therapeutic doses.

MATERIALS AND METHODS

Reagents and chemicals

The solvents used in the extraction procedure were spectral grade methylene chloride and reagent grade diethyl ether. Both the solvents were distilled in glass over sodium wire. The diethyl ether was stored in actinic flasks containing sodium wire to prevent peroxide formation and moisture absorption.

Metronidazole was supplied by Delmar Chemicals, (Lachine, Canada) and 1-β-hydroxyethyl-2-methoxy-5-nitroimidazole (II) used as an internal standard was generously donated by Dr. B. Senkowski, Hoffman-La Roche Inc., Nutley, N.J., U.S.A.

Tri-Sil concentrate and silylation grade acetonitrile (Pierce, Rockford, Ill., U.S.A.) were obtained through Chromatographic Specialities (Brockville, Canada).

Stock solutions

Aqueous solutions of I and II (100 μg/ml) were freshly prepared each week and refrigerated. In experiments to find the extraction limits, the solutions of I and II were made in ethyl acetate immediately before use.

Gas–liquid chromatography

A Perkin-Elmer Model F11 gas chromatograph equipped with a hydrogen flame ionization detector and a Perkin-Elmer, Coleman Model 196 recorder was employed. The chromatographic column consisted of spiral glass tubing, 6 ft. $\times$ 1.5 mm I.D., packed with mixed liquid phases, *i.e.* 1% Apiezon L, 0.5% SE-30 ultra phase, and 0.5% OV-7 on 80–100 mesh Chromosorb W. The column was conditioned by maintaining the column at 300° for 24 h with low nitrogen flow and injecting Silyl-8. Operating temperatures for metronidazole analysis were: column, 135°; injection port, 210°; detector, 210°. The carrier gas was nitrogen at a flow-rate of 80 ml/min. The air–hydrogen mixture was adjusted to get a maximum response. The chart speed was 5 mm/min.

Gas–liquid chromatography–mass spectrometry

All the mass spectra were recorded using a Hitachi Perkin-Elmer Model RMS4 mass spectrometer with a Perkin-Elmer gas chromatograph Model 990 coupled through a two-stage jet separator interphase.

General procedure

1.0 ml of the plasma containing I was added to 1 ml of aqueous solution of II (15 μg/ml) in a centrifuge tube (42 ml) to which 4.0 ml of 0.1 *M* carbonate–bicarbonate buffer (pH 9.0) were then added. The sample was shaken on a vortex mixer for 30 sec and 15.0 ml of a mixture of diethyl ether and methylene chloride (14:11)

were added. The centrifuge tube was tightly stoppered and was shaken (216 cycles/min) on a flat bed shaker (Eberbach, Ann Arbor, Mich., U.S.A.) at an angle of 30° for 30 min. 13.0 ml of the upper organic layer were pipetted into a conical glass stoppered tube (45 ml). The extraction was repeated twice with two 15.0-ml portions of the solvent mixture. Each time 15.0-ml portions were removed and combined with the previous extract. The combined extracts were evaporated to a volume of approximately 1 ml on a water-bath at 60° under a stream of dry nitrogen. A small crystal of anhydrous calcium chloride was added and the tube was swirled gently. The concentrated extract was then transferred quantitatively to a glass-stoppered conical tube (3 ml) by means of a pasteur pipette with the aid of two 0.5-ml portions of methylene chloride. The combined washings and extracts were then evaporated to dryness in a water-bath at 60° under a stream of dry nitrogen.

Preparation of trimethylsilyl ethers of metronidazole and internal standard

Trimethylsilyl (TMS) derivatives were prepared by adding 50 μl of acetonitrile and 30 μl of Tri-Sil concentrate to the evaporated extracts in the tube. The tube was stoppered, mixed for 30 sec on a vortex mixer and was left overnight at room temperature. The content was evaporated to dryness at room temperature under a stream of nitrogen; 50 μl of acetonitrile were added carefully in small portions and the solid residue was redissolved by mixing on the vortex mixer for 1 min after which 1–2 μl of the sample was injected into the chromatograph. The retention time of the TMS ether of I was 4.9 min under the conditions described.

Calibration curves and quantitation

A standard calibration curve was established by adding I in the range of 0.25 to 16.0 μg/ml to fresh heparinized plasma and proceeding as described above in the general procedure for plasma. Quantitation was by means of the ratio of the peak height of I to that of II. A standard curve constructed by plotting the peak height ratio (Y) against the amount (μg added) of I (x) (Table I) coincided with the line $Y = mx$, where the slope (m) had a value of 0.200 $\pm$ 0.001.

Drug administration for plasma samples

A tablet (250 mg) of I was administered orally to a healthy male volunteer

TABLE I

ESTIMATION BY GLC OF METRONIDAZOLE (I) ADDED TO PLASMA

C.V. = coefficient of variation.

Amount of I added (μg)	*Mean peak height ratio compd. I/II*	*No. of estimations*	*C.V.*
0.25	0.05	8	6.23
0.50	0.11	4	9.52
1.0	0.19	4	4.30
2.0	0.42	5	4.02
4.0	0.78	6	1.04
8.0	1.60	3	1.91
16.0	3.21	4	0.97
		mean	4.00

(71 kg) and nine venous blood samples (10 ml) were withdrawn from the cubital vein by means of heparinized vacutainers during the 24 h following ingestion.

Venous blood samples were also collected from cephalic vein of a male dog (21 kg) who was administered perorally a capsule containing 250 mg of I. Blood samples were centrifuged immediately after collection and the collected plasma was either analysed the same day or stored in a freezer (−15°) and analysed later.

RESULTS AND DISCUSSION

The ultraviolet (UV) absorption spectra of I and II determined in ethyl acetate showed absorption maxima at 322.5 nm and at 339 nm, respectively. The specific absorptivity was sufficient to quantitate 100 μg of each compound per milliliter of final solution. The determination of the extractability of these compounds into organic solvents from plasma buffered at different pH values was effected initially using the UV absorption of I in ethyl acetate for quantitation. It was determined that using a mixture of methylene chloride and diethyl ether (11:14) gave better than 80% recovery of metronidazole from plasma buffered to pH 9.0. The mixed solvents were used in order to have the organic layer on top for routine analysis. The recovery declined significantly at pH values less than 9.0. No major cleanup was required as no interfering peaks were detected on GLC (Fig. 1a).

Initial attempts to analyse I without derivatization revealed poor response characteristics with a minimum detectability in the 2–5-μg range. Appreciable tailing

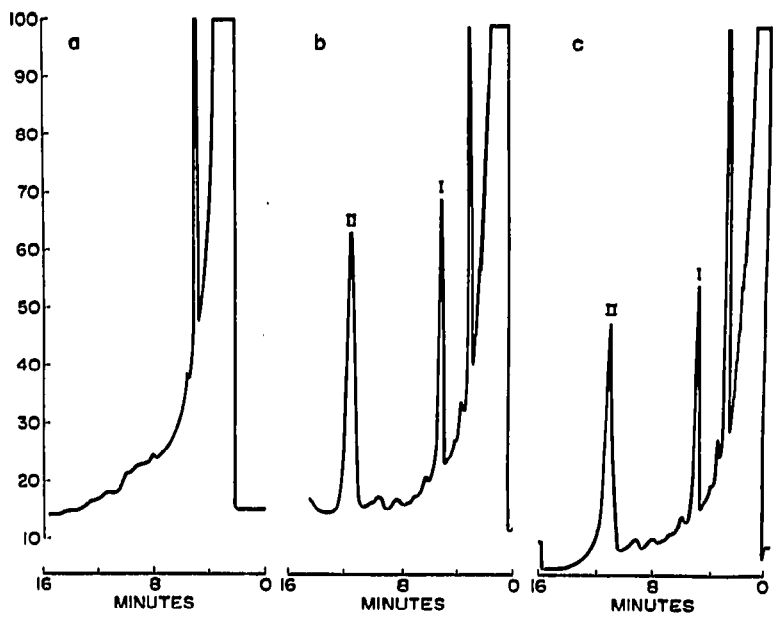

Fig. 1. Chromatograms of human plasma extracts. (a) Blank plasma; (b) control plasma to which 5.0 μg of I and 15.0 μg of II were added; (c) plasma (containing ≃ 4.9 μg of I) from a human volunteer who received an oral dose of I; II (15.0 μg) was added directly to the plasma and the extract prepared. Retention times are: I, 4.9 min; and II, 11.1 min. GLC conditions are described in Materials and methods.

of peaks was observed on liquid phases such as Carbowax 20M, SE-30, Apiezon L, OV-17 and OV-7. It was assumed that the polar hydroxyl present in the molecule of I was interacting with the liquid phases to cause tailing. Silylation decreased the tailing of peaks considerably. Furthermore, use of a column with mixed liquid phases resulted in much sharper peaks for the TMS ethers of I and II and much better detector response and hence greater sensitivity. In order to establish the structures of TMS ethers, eluates from the GLC column (mixed phases) were fed directly into the mass spectrometer and mass spectra of TMS ethers of I and II (Fig. 2) were recorded.

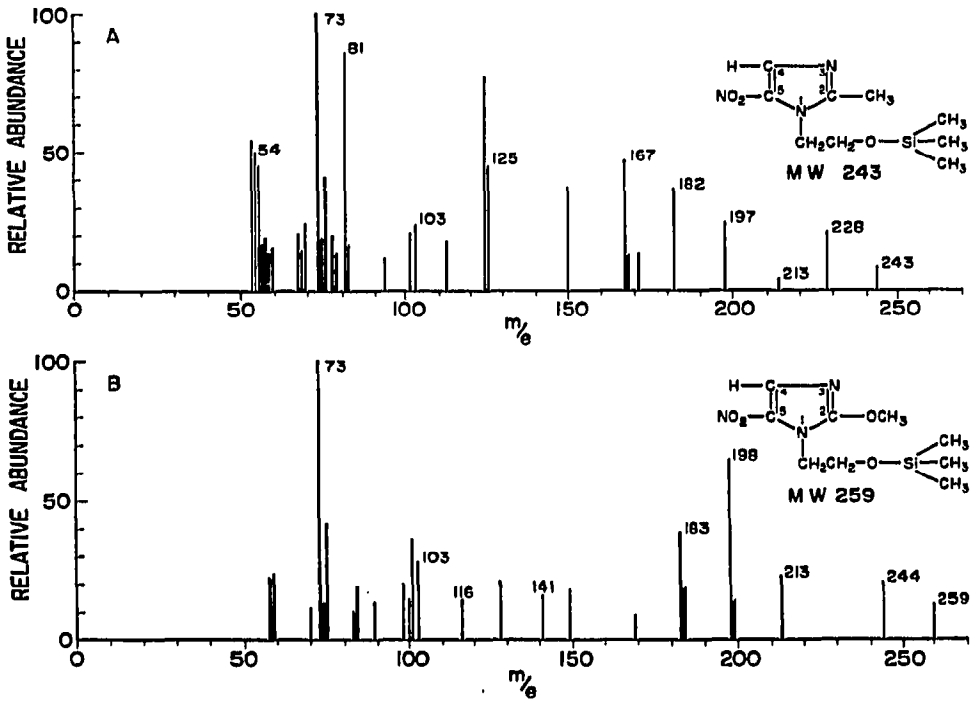

Fig. 2. GLC–mass spectra of TMS ethers of metronidazole (A) and internal standard (B).

The mass spectrum (Fig. 2A) of TMS ether of I had a molecular ion of *m/e* 243 for ion ia and base peak at *m/e* 73 for ion ii. Diagnostic ions were located at *m/e* 228 (ion iiia), *m/e* 213 (ion iv), *m/e* 197 (ion va), *m/e* 182 (ion via), *m/e* 167 (ion viia), *m/e* 125 (ion viiia), *m/e* 103 (ion ix), *m/e* 81 (ion x) and *m/e* 54 (ion xi).

The mass spectrum (Fig. 2B) of TMS ether of II gave a molecular ion at *m/e* 259 (ion ib) and a base peak at *m/e* 73 (ion ii). The ions at *m/e* 244, *m/e* 213, *m/e* 198, *m/e* 183, and *m/e* 103 have been postulated as structures iiib, vb, vib, viib, and ix. The ion at *m/e* 116 (structure xii) is formed by the McLafferty rearrangement.

Optimum conditions for the concomitant silylation of both compounds (I and II) were obtained in acetonitrile as solvent with a reaction time of 10 min at room temperature for quantitative reaction. It is essential that the reaction mixture be mixed vigorously on vortex mixer after the addition of Tri-Sil concentrate. The TMS derivatives were found to be stable and can be analysed on the next day. The extract

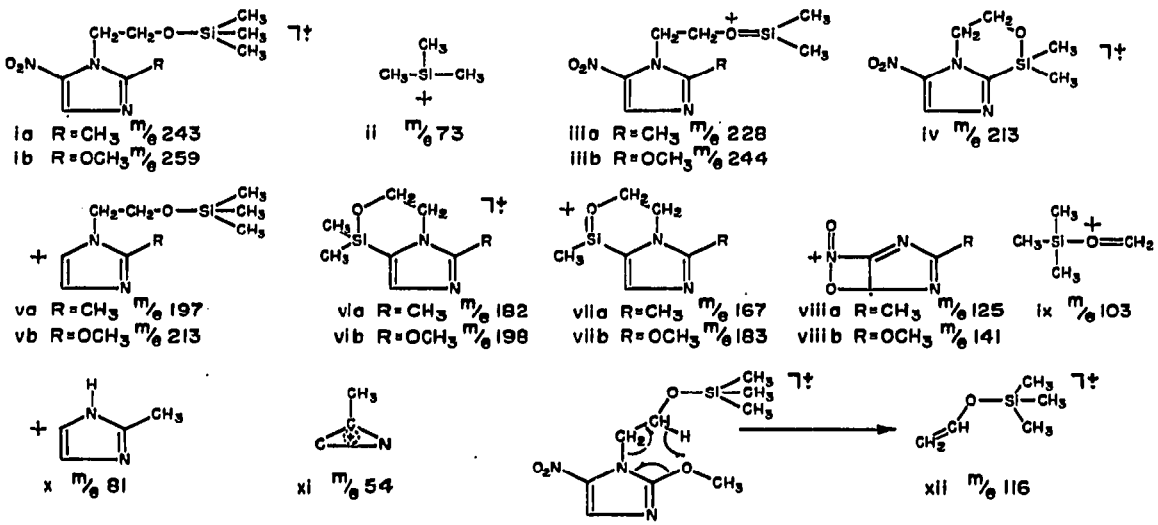

Fig. 3. Mass spectral fragmentation of TMS ethers of I and II.

residue as well as extracts can also be left overnight before formation of derivatives the following day. The peak height ratio of the two was used as the index of detector performance and overall efficiency of the analytical procedure. Thus the external standard curve from the solvent and the plasma-recovered internal standard curve, constructed by plotting the peak height response ratios of TMS-I/TMS-II *versus* concentration of I containing a constant amount of II as the reference standard, were comparable. The overall recoveries of 4–8-μg amounts of compounds I and II from plasma were of the order of 101.69 $\pm$ 1.46 and 98.66 $\pm$ 4.73%, respectively (Table II). The reproducibility of the calibration curve of peak height ratio I/II *vs.* concentration of I is indicated in Table I. The mean coefficient of variation from 0.25–16 μg is 4%.

The GLC assay applied to the determination of plasma levels of I in dog gave

TABLE II

RECOVERY OF METRONIDAZOLE (I) AND 1-β-HYDROXYETHYL-2-METHOXY-5-NITROIMIDAZOLE (II) FROM PLASMA BY GLC ASSAY

Amount added (μg)	*Amount recovered (μg)*	*Recovery (%)*
Recovery of I		
4.0	4.06	101.50
4.0	3.94	98.50
8.0	8.11	101.38
8.0	8.11	101.38
	Mean	100.69 $\pm$ 1.46
Recovery of II		
4.0	3.98	99.50
4.0	4.16	104.00
8.0	7.89	98.63
8.0	7.40	92.50
	Mean	98.66 $\pm$ 4.73

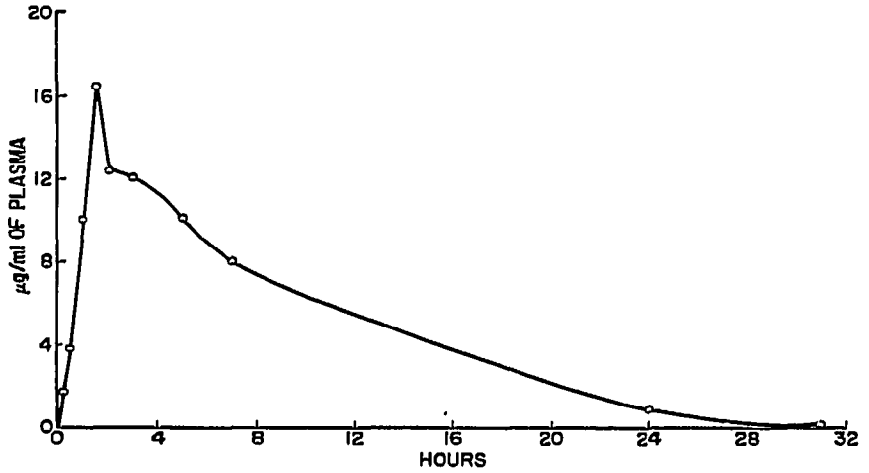

Fig. 4. Plasma levels of metronidazole measured in a dog (21 kg) following the administration of a single 250-mg oral dose of metronidazole.

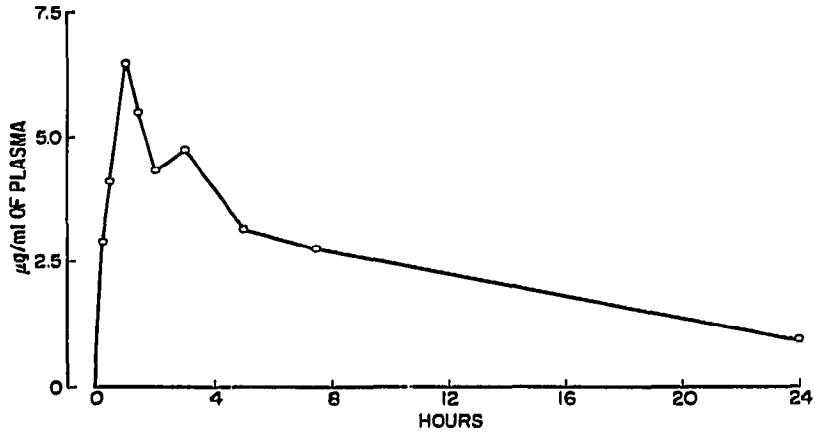

Fig. 5. Plasma levels of metronidazole measured in a man (71 kg) following the administration of a single 250-mg oral dose of metronidazole.

results which are shown in Fig. 4. Repeat assays were within 5% even at the 1.0 µg/ml level.

The plasma decay of I following administration of 250 mg to a human subject is shown in Fig. 5. Repeat assays were within 7% absolute error in this therapeutic range. Additional estimations for the 3-h blood sample indicated that this is a true value perhaps attributable to biliary excretion.

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