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ELECTRON-CAPTURE GAS CHROMATOGRAPHIC ASSAYS OF 5-NITROIMIDAZOLE CLASS OF ANTIMICROBIALS IN BLOOD*

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SUMMARY

Sensitive and selective electron-capture gas chromatographic methods for the determination of N-1-substituted 5-nitroimidazole class of antiprotozoals from blood are described. Metronidazole, secnidazole and ornidazole having a hydroxyl function in the N-1 substitution, were converted to their respective trimethylsilyl derivatives before chromatography on an OV-11 column. Tinidazole and satranidazole, devoid of the hydroxy group but containing a sulphur atom in the molecule, were chromatographed as such on the same stationary phase. Blood levels as low as 50 ng/ml for all the 5-nitroimidazoles have been measured with good precision. The methods can be readily utilized for pharmacokinetic studies.

INTRODUCTION

The advent of metronidazole and other N-1-substituted 5-nitroimidazoles like secnidazole, ornidazole, tinidazole and satranidazole** (Fig. 1) has revolutionized the chemotherapy of protozoal and anaerobic bacterial infections [1]. Barring their broad similarity, the compounds differ markedly with respect to their pharmacokinetic characteristics and, hence, in dosage regimens. However, different analytical methods have been employed for comparative studies. These include bioassay [2], absorptiometry [3], polarography [4], "flying-spot" thin-layer chromatographic (TLC) densitometry [5], high-performance liquid chromatography [6—9] and gas chromatography with flame ionization detection [10—12].

In this paper we report two sensitive and selective electron-capture gas chro-

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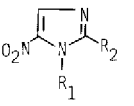


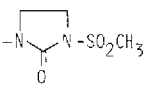
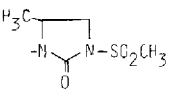
		R_1	R_2
I	METRONIDAZOLE	$-CH_2CH_2OH$	$-CH_3$
	SECNIDAZOLE	$-CH_2CHOHCH_3$	$-CH_3$
	ORNIDAZOLE	$-CH_2CHOHCH_2Cl$	$-CH_3$
II	TINIDAZOLE	$-CH_2CH_2SO_2CH_2CH_3$	$-CH_3$
	SATRANIDAZOLE	$-CH_3$	
	GO 11386	$-CH_3$	

Fig. 1. Structural formulae of N-1-substituted 5-nitroimidazoles assayed with (I) and without (II) derivatization procedure.

matographic procedures: one for the analysis of 5-nitroimidazoles with a hydroxyl function in the N-1 substitution (metronidazole, secnidazole and ornidazole), and the other for compounds without the hydroxy group but containing a sulphur atom in the molecule (tinidazole and satranidazole). Both methods are devoid of elaborate extractions, wash procedures and TLC clean-up steps, and utilize a single column and liquid stationary phase. The methods are easily reproducible and hence suitable for routine analysis of blood for pharmacokinetic studies following therapeutic doses of 5-nitroimidazoles.

EXPERIMENTAL

Chemicals and reagents

Spectroscopic grade methanol (Uvasol) and reagent grade methylene chloride, toluene and cyclohexane were obtained from E. Merck (Bombay, India). Diethyl ether was obtained from Hyderabad Chemicals and Pharmaceuticals (Hyderabad, India). Methylene chloride and diethyl ether were freshly distilled on a 120-cm Vigreux column before use. Toluene and cyclohexane were thoroughly washed successively with concentrated sulphuric acid, water, 1 *M* sodium hydroxide and water, dried over calcium chloride and distilled on a 120-cm Vigreux column.

The derivatization reagent, N,O-bis-(trimethylsilyl)-trifluoroacetamide (BSTFA) was obtained from Pierce Chemical Company (Rockford, IL, U.S.A.), and the stationary phase (3% OV-11 on 100—120 mesh Supelcoport) was from

Supelco (Bellefonte, PA, U.S.A.). Ultra pure nitrogen (Iolar-2) from Indian Oxygen (Bombay, India), served as the carrier gas.

All the 5-nitroimidazoles mentioned in this study were synthesized in-house.

Synthesis of trimethylsilyl derivatives

A mixture of 1-(hydroxyalkyl)-2-methyl-5-nitroimidazole (3 mmol) and N,O-bis-(trimethylsilyl)-trifluoroacetamide (1 ml) was stirred at 25—27°C for 10 h under anhydrous conditions. Excess reagent was then evaporated off by heating at 60—70°C at 2—3 mm Hg for 3 h to afford the trimethylsilyl (TMS) derivative. TLC (silica; chloroform—methanol, 96:4, v/v) comparison with starting material showed complete conversion.

TMS derivative of metronidazole. Analysis: found C, 44.41; H, 7.25; N, 17.58. $C_9H_{17}N_3O_3Si$ (mol. wt. 243) requires C, 44.42; H, 7.04; N, 17.27. ^{1}H-NMR* (C^2HCl_3): δ 7.97 (1H, s, C-4H), 4.47 (2H, t, N-CH_2), 3.90 (2H, t, O-CH_2), 2.50 (3H, s, 2-CH_3) and 0 ppm [9H, s, Si$(CH_3)_3$]. MS: 243 (M^+ at *m/z*).

TMS derivative of secnidazole. Analysis: found C, 46.96; H, 7.71; N, 16.73. $C_{10}H_{19}N_3O_3Si$ (mol. wt. 257) requires C, 46.67; H, 7.44; N, 16.33. ^{1}H-NMR (C^2HCl_3): δ 8.05 (1H, s, C-4H), 4.60 (1H, d with fine structure, 1H of N-CH_2), 3.9—4.2 (2H, complex m, $CHCH_3$ + 1H of N-CH_2), 2.62 (3H, s, 2-CH_3), 1.37 (3H, d, $CHCH_3$) and 0 ppm [9H, s, Si$(CH_3)_3$]. MS: 257 (M^+ at *m/z*).

TMS derivative of ornidazole. m.p. 47—50°C. Analysis: found C, 41.42; H, 6.53; N, 14.06. $C_{10}H_{18}ClN_3O_3Si$ (mol. wt. 293) requires C, 41.16; H, 6.21; N, 14.40. ^{1}H-NMR (C^2HCl_3): δ 8.05 (1H, s, C-4H), 4.80 (1H, d with fine structure, 1H of N-CH_2), 4.13—4.60 (2H, complex m, $CHCH_2Cl$ + 1H of N-CH_2), 3.65 (2H, d, CH_2Cl), 2.63 (3H, s, 2-CH_3) and 0 ppm [9H, s, Si$(CH_3)_3$]. MS: 293 (M^+ at *m/z*).

Internal standards

In procedure I, ornidazole served as the internal standard for both metronidazole and secnidazole, while metronidazole was used as the internal standard for ornidazole assays. In procedure II, Go 11386 served as the internal standard for the assays of both tinidazole and satranidazole.

Procedure I (metronidazole, secnidazole and ornidazole)

One milligram each of metronidazole, secnidazole and ornidazole was dissolved separately in 100 ml of methanol to yield stock solutions of 10 μg/ml concentration. Aliquots corresponding to 100, 200, 300 and 400 ng of each compound and 200 ng of appropriate internal standard were pipetted into 15-ml standard joint silanized glass tubes; 1.0 ml of human blood was added and the tubes were vortexed and allowed to stand for 5 min at room temperature for equilibration. Then 0.1 ml of 1 *M* sodium hydroxide was added and the tubes vortexed again; 2 ml of a mixture of methylene chloride—diethyl ether (11:14, v/v) were added, the tubes were stoppered with a water seal and extraction was carried out on a reciprocal shaker for 5 min at full speed. The tubes

*NMR chemical shifts are with reference to TMS as the external standard.

were centrifuged at 4000 *g* for 5 min and the organic layer was transferred to a 10-ml conical glass tube. The extraction was repeated and the organic layers pooled and evaporated to dryness at 37°C under a gentle stream of nitrogen. The sides of the tubes were rinsed with 0.5 ml of the solvent mixture and re-evaporated to near dryness.

Derivatization was now carried out by adding 20 μl* of BSTFA reagent; the tubes were gently swirled and allowed to stand at room temperature (26°C) for 1 h. Excess reagent was then evaporated to dryness at 37°C under nitrogen and the TMS ester reconstituted in 1 ml of cyclohexane; 2—3 μl were injected into the gas chromatograph.

Procedure II (tinidazole and satranidazole)

Stock standard solutions of tinidazole, satranidazole and Go 11386 (internal standard) were prepared in methanol to yield concentrations of 10 μg/ml. Working standards (2 μg/ml) of tinidazole and satranidazole were prepared by a five-fold dilution of the stock solution, while that of Go 11386 (1 μg/ml) was prepared by a ten-fold dilution with methanol. Aliquots corresponding to 100, 200, 300 and 400 ng of tinidazole or satranidazole and 50 ng of internal standard were pipetted into 15 ml-standard joint silanized glass tubes; 1.0 ml of human blood was added and the tubes were vortexed and allowed to stand for 5 min. Then 0.1 ml of 1 *M* sodium hydroxide was added and tubes were vortexed again. A single extraction with 4 ml of methylene chlorode—diethyl ether (11:14, v/v) mixture was carried out on a reciprocal shaker for 5 min. The tubes were centrifuged at 4000 *g* for 5 min. The organic phase was separated, evaporated to near dryness under nitrogen and reconstituted in 0.5 ml of distilled toluene; 2—3 μl of the solvent were injected directly into the gas chromatograph.

Gas chromatography

The gas chromatograph consisted of a Pye-Unicam Model 204 instrument equipped with a 10-mCi ^{63}Ni electron-capture detector. For all the 5-nitroimidazoles in the two procedures, chromatography was carried out on a pre-

TABLE I

CHROMATOGRAPHIC CONDITIONS

Compound	Column	Gas flow-rate (ml/min)	Temperature (°C)		
			Column	Injector	Detector
Metronidazole, Secnidazole, Ornidazole	3% OV-11	50	180	150	250
Tinidazole	3% OV-11	45	260	200	300
Satranidazole	3% OV-11	40	280	220	300

*50 μl for secnidazole concentrations of 300 ng/ml and above.

conditioned 150 cm × 4 mm I.D. glass column packed with 3% OV-11 on 100—120 mesh Supelcoport. Other chromatographic conditions are summarized in Table I.

All injections were performed on-column using a 10-μl Hamilton microsyringe with a 10-cm needle. The detector attenuation was set at 64 × 5 and chromatograms were recorded on an Omniscribe recorder (10 mV) at a chart-speed of 0.5 cm/min. Peak heights were measured and standard/internal standard height ratios were used for calibration and quantitation.

RESULTS

Resolution

Under the chromatographic conditions described in Table I, TMS derivatives of metronidazole and secnidazole were well resolved from that of ornidazole — the internal standard. Tinidazole and satranidazole were also well separated from the internal standard Go 11386. The chromatograms of synthetic TMS esters of metronidazole, secnidazole and ornidazole and of standards of tinidazole, satranidazole and Go 11386 are presented in Fig. 2. The retention times of all the 5-nitroimidazoles are listed in Table II. The internal standard Go 11386 exhibits a retention time of 5.0 min when used with tinidazole and 2.8 min under the conditions of satranidazole assay.

Reaction kinetics and recoveries

The optimal conditions for derivatization were determined by incubating BSTFA reagent with an extract of blood spiked with 200 ng/ml of

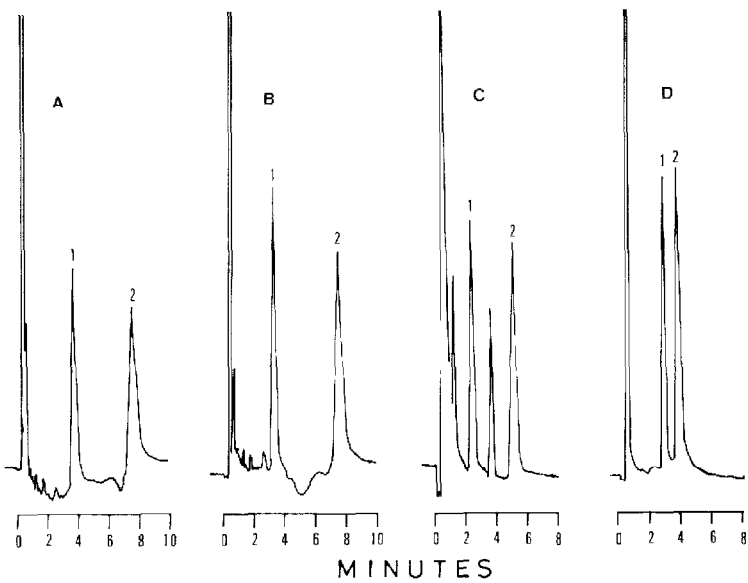


Fig. 2. Chromatograms of synthetic standards of: (A) 400 pg of TMS-metronidazole (1) and 400 pg of TMS-ornidazole (2); (B) 500 pg of TMS-secnidazole (1) and 500 pg of TMS-ornidazole (2); (C) 800 pg of tinidazole (1) and 400 pg of Go 11386 (2); and (D) 200 pg of Go 11386 (1) and 500 pg of satranidazole (2).

TABLE II

RETENTION TIMES OF 5-NITROIMIDAZOLES ON 3% OV-11 COLUMN

Chromatographic conditions are given in Table I.

Compound	Retention time (min)
Metronidazole	3.6
Secnidazole	3 4
Ornidazole	7.8
Tinidazole	2.4
Satranidazole	3.8
Go 11386	2.8*, 5.0**

*Under the conditions of satranidazole assay.
**Under the conditions of tinidazole assay

TABLE III

CHROMATOGRAPHIC PRECISION AND RECOVERIES FROM SPIKED BLOOD SAMPLES

Results are based upon six within-day replicate injections

Compound	Spiked concentration (ng/ml)	Precision (C.V., %)	Recovery (%)
Metronidazole	200	3.2	82
Secnidazole	200	5.7	73
Ornidazole	200	3 1	81
Tinidazole	250	2 2	81
Satranidazole	250	1.8	97
Go 11386	100	—	89

hydroxylated nitroimidazoles for 0.5—1.5 h at room temperature or 50°C and comparing peak heights with those of synthetic TMS ester standards. Reactions were generally complete by 1.0 h at room temperature for all the three nitroimidazoles. Prolonged incubation times under our conditions (relative humidity > 60%) resulted in lower recoveries, especially of secnidazole. TMS esters are well known to be unstable in the presence of moisture.

Recoveries for the entire procedure, with a 1-h derivatization period, were 82, 73 and 81% for metronidazole, secnidazole and ornidazole, respectively. In procedure II, where only a single extraction step is involved, the recoveries for tinidazole, satranidazole and Go 11386 were 81, 97 and 89%, respectively (Table III).

Chromatographic precision

Six replicate injections of spiked blood samples submitted to the full procedures were carried out to check within-day chromatographic precision. The results are recorded in Table III. The coefficient of variation (C.V.) was within 5.7% for all the compounds.

Linearity

The linearity of the methods was established with human blood calibration curves in the concentration range 100—400 ng/ml based on a 1-ml sample volume. Table IV lists the regression equations and correlation coefficients obtained with 6—8 replicate analyses for each concentration. Correlation coefficients were obtained in the range 0.9951—0.9998, indicating excellent linearity of the two procedures for all the 5-nitroimidazoles tested. Typical chromatograms are shown in Figs. 3 and 4.

Accuracy and precision

The methods were applied to determine concentrations of spiked blood samples in the range of 50—450 ng/ml for assessing the accuracy and precision of the procedures. Table V records the mean values and C.V. Results indicate

TABLE IV

REGRESSION EQUATIONS FOR HUMAN BLOOD CALIBRATION CURVES

Compound	Concentration range (ng/ml)	n*	Equation ($Y = a + bX$)	Correlation coefficient (r)
Metronidazole	100—400	8	$Y = -0.055 + 0.0071X$	0.9998
Secnidazole	100—400	8	$Y = 0.095 + 0.0074X$	0.9951
Ornidazole	100—400	8	$Y = 0.070 + 0.0027X$	0.9988
Tinidazole	100—400	8	$Y = 0.035 + 0.0062X$	0.9993
Satranidazole	100—400	6	$Y = -0.080 + 0.0059X$	0.9979

*Number of analyses per concentration

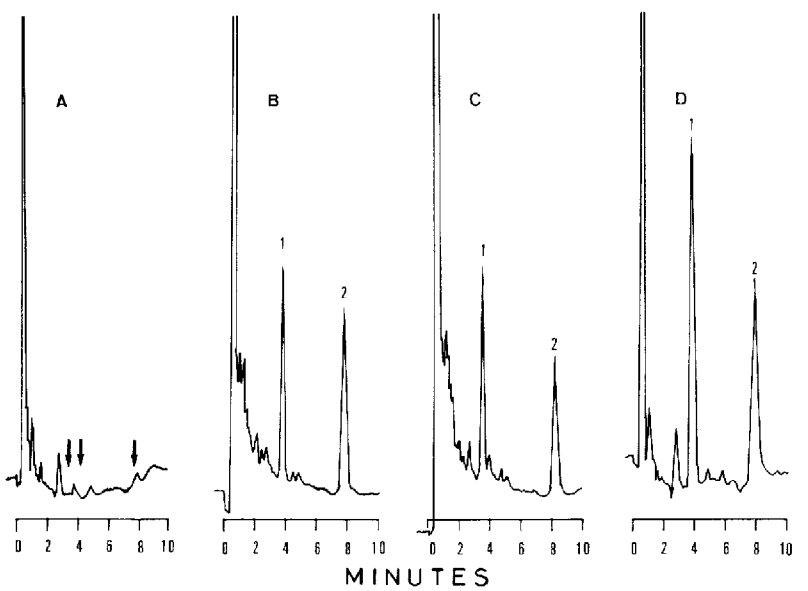


Fig. 3. Chromatograms of extracts: (A) human blood blank; (B) blood spiked with 200 ng of metronidazole (1) and 200 ng of ornidazole (2); (C) blood spiked with 200 ng of secnidazole (1) and 200 ng of ornidazole (2); and (D) blood sample of a volunteer receiving 200 mg of metronidazole per os, metronidazole 4.61 μg/ml (1) and ornidazole (2).

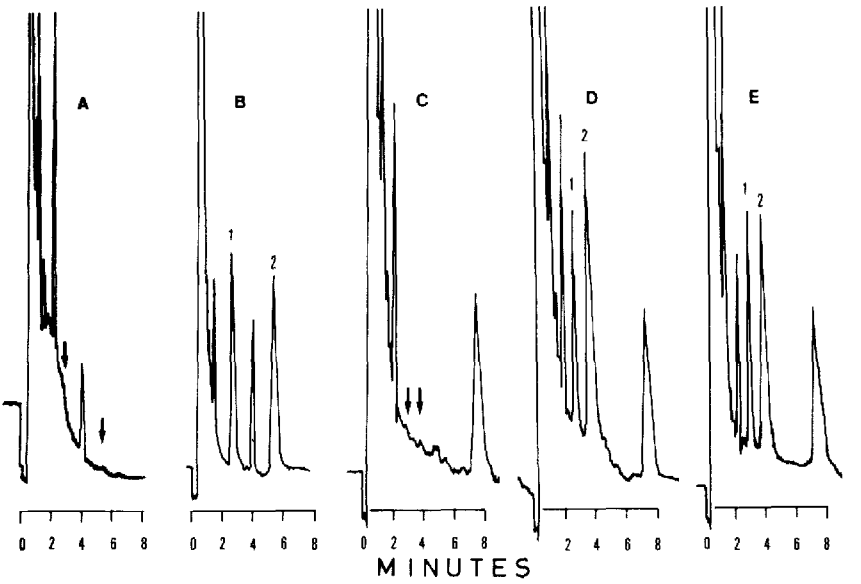


Fig. 4. Chromatograms of extracts of: (A) human blood blank; (B) blood spiked with 200 ng of tinidazole (1) and 100 ng of Go 11386 (2); (C) human blood blank (for satranidazole assay); (D) blood spiked with 50 ng of Go 11386 (1) and 200 ng of satranidazole (2); and (E) blood sample of a volunteer receiving 200 mg of satranidazole per os, Go 11386 (1) and satranidazole 4 μg/ml (2).

TABLE V

PRECISION AND ACCURACY OF THE PROCEDURES APPLIED TO SPIKED HUMAN BLOOD SAMPLES

Data represent concentrations found (ng/ml) ± C.V. (%). Each value represents the mean of four determinations.

Compound	Concentration spiked (ng/ml)			
	50	150	250	450
Metronidazole	53.7 ± 9.4	160.3 ± 5.7	255.2 ± 3.3	445.2 ± 2.6
Secnidazole	52.3 ± 5.1	148.6 ± 4 1	241.0 ± 2.0	444.2 ± 3.8
Ornidazole	56.5 ± 3.3	146.4 ± 9.7	253.0 ± 6.9	444.6 ± 3.7
Tinidazole	54.8 ± 5.0	145 6 ± 2.2	252.6 ± 1.8	442.1 ± 3.1
Satranidazole	48.9 ± 2.7	147 7 ± 5.6	250.0 ± 5.9	451.1 ± 5.1

that levels in the above range can be estimated with good accuracy and precision. The C.V. for a concentration of 50 ng/ml ranged from 2.7% for satranidazole to 9.4% for metronidazole.

Application

Procedure I has been used to measure the blood levels of metronidazole following a single oral dose of 200 mg to a healthy volunteer. A C_{max} of 4.61 μg/ml was recorded at 1 h and a level of 0.15 μg/ml was detected at 48 h (Fig. 5). Under the conditions of assay, the known metabolite of metronidazole

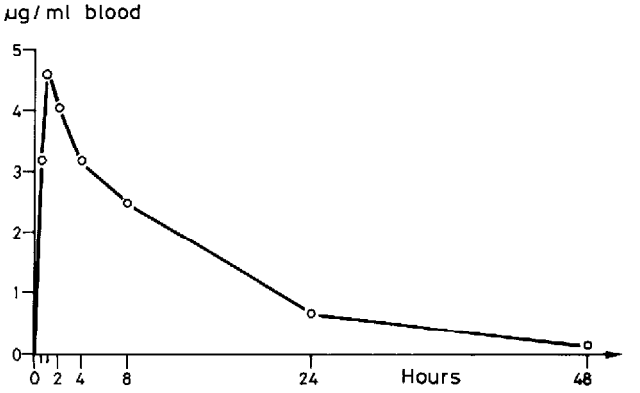


Fig. 5 Blood concentrations of metronidazole after an oral dose of 200 mg of drug to a human volunteer

[viz. 1-(2-hydroxyethyl)-2-hydroxymethyl-5-nitroimidazole] which would be amenable to derivatization, did not interfere with the analysis of the parent compound (Fig. 3D). Similarly, in the analysis of satranidazole, from the blood of a volunteer receiving a 200-mg dose of the drug, no interference from metabolites was noticed (Fig. 4E).

DISCUSSION

In procedure I the presence of traces of moisture prior to derivatization led to erratic results, probably due to hydrolysis of the BSTFA reagent or the TMS ester. Care was taken to carefully transfer solvent extracts free from traces of lower aqueous layer, evaporating the extracts not to complete dryness but until a residual film was obtained. BSTFA reagent was then added. Similarly, prior to reconstitution in cyclohexane in procedure I or toluene in procedure II, complete evaporation to dryness under nitrogen was avoided as this resulted in losses due to adsorption on the glass surface.

While derivatization of metronidazole, secnidazole and ornidazole is necessary to decrease column adsorption and increase volatility and sensitivity, for tinidazole and satranidazole — analysed without derivatization — higher temperatures were needed for volatilization. However, the presence of the sulphur atom would lend itself to the enhanced electron-capture sensitivity of these two compounds.

The only other electron-capture gas chromatographic method reported is for a single 5-nitroimidazole, namely ornidazole [13]. The method has an elaborate extraction procedure followed by a TLC clean-up step which involves, first, separation of the external standard from the internal standard and, later, elution of the combined spots of the two standards. Further, the derivatization is effected by the use of two reagents. The methods described in this paper overcome such problems in having a conventional internal standard and a simple extraction procedure free of an additional clean-up step. Moreover, using a single column and stationary phase the quantitative analysis of five different 5-nitroimidazoles from blood, following therapeutic doses, is possible.

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