Biochemical Pharmacology, Vol. 20, pp. 339–349. Pergamon Press, 1971. Printed in Great Britain

STUDIES ON ANTIPROTOZOANS—III

ISOLATION, IDENTIFICATION AND QUANTITATIVE DETERMINATION IN HUMANS OF THE METABOLITES OF A NEW TRICHOMONACIDAL AGENT

P. N. GIRALDI, G. P. TOSOLINI, E. DRADI, G. NANNINI, R. LONGO, G. MEINARDI, G. MONTI and I. DE CARNERI

Department of Chemistry and Department of Microbiology, Instituto Carlo Erba per Ricerche Terapeutiche, Milano, Italy

(*Received* 28 *May* 1970; *accepted* 8 *July* 1970)

Abstract—The metabolism has been investigated in man of 1-(*N*-β-ethylmorpholine)-5-nitroimidazole, a new trichomonacidal agent.

From the urine of patients treated with the compound, two main metabolites have been isolated, quantitatively determined and their chemical structure established. The techniques used for these purposes have also been described.

Both metabolites are active on *Trichomonas vaginalis*.

BOTH the synthesis and the trichomonacidal activity of a series of styryl-1-alkylamino-nitroimidazole and 1-alkylamino-nitroimidazole derivatives have been described in previous papers,[1,2] where the motives leading to this present research have also been illustrated.

1-(*N*-β-ethylmorpholine)-5-nitroimidazole has resulted to be the most active and interesting compound: it has been given the generic name of Nitrimidazine.* The *in vitro* and *in vivo* activities[3,4] have been fully confirmed by extensive clinical trials carried out by oral and intravaginal administration.[5–8]

It is interesting to point out that satisfactory blood levels have also been provided by vaginal administration, a finding obviously indicative of a satisfactory topical absorption.

Moreover this compound is highly active in human giardiasis, intestinal and hepatic amebiasis.

Consequently we thought it interesting to initiate a metabolic investigation of this new compound in humans. In this study we present the methods used for the isolation, identification and quantitative determination of urinary metabolites as well as the description of the methods of synthesis used for their preparation.

MATERIALS AND METHODS

(a) *Isolation and identification of urinary metabolites. Metabolite I*

Human subjects are given 500 mg of Nitrimidazine per os. Urine is collected for 48-hr period.

* The WHO proposed name for Nitrimidazine is Nimorazole.

The urine is adjusted at pH 8, extracted three times with methylene chloride; this extract is washed first with 5% $NaHCO_3$, then with water, concentrated to a small volume and chromatographed on silica gel.

When various running phases are used, two spots are separated which give positive reaction for the nitrogroup on the imidazol ring and whose $R_f \times 100$ values are reported in Table 1.

TABLE 1. $R_f \times 100$ VALUES OF NITRIMIDAZINE AND ITS METABOLITES

Running phase	A	B	C	D
Nitrimidazine	92	36	32	37
Spot I	92	36	32	37
Spot II	75	32	12	22

TLC on silica gel; Running phase:
A = $CHCl_3$–CH_3OH–NH_4OH (150:30:5)
B = CH_3COCH_3
C = C_6H_6–CH_3COCH_3–$(C_2H_5)_2NH$ (170:20:10)
D = C_6H_6–CH_3COCH_3–$(C_2H_5)_2NH$ (100:100:5).

One of these spots (Spot I) shows the same $R_f \times 100$ value of Nitrimidazine by all running phases used.

To investigate the nature of compound II, the CH_2Cl_2 fraction has been subjected to chromatographic separation on a silica gel column. Mixture C has been used as running phase.

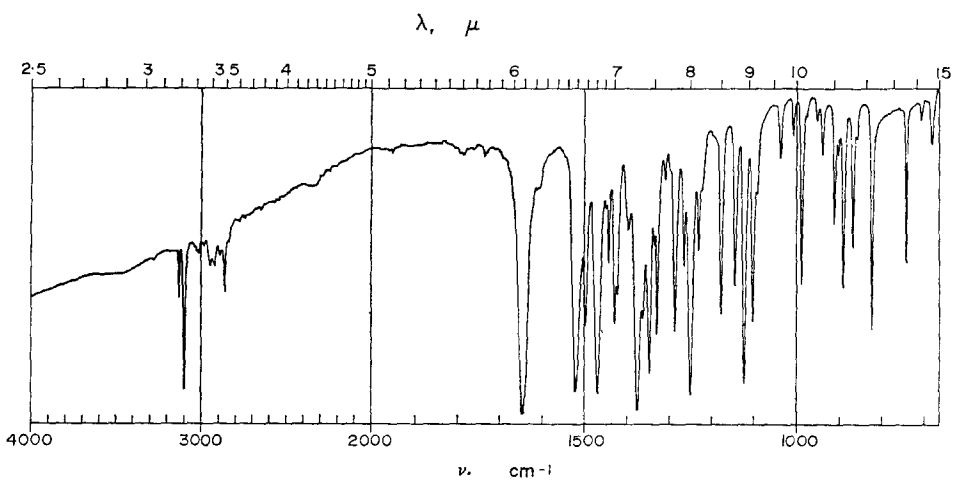


FIG. 1. Infrared spectrum in KBr of Metabolite I.

Concentration to dryness is carried out only of those eluted fractions in which the presence of compound II has been verified by TLC.

Metabolite I is thus isolated as a pale yellow powder and crystallized from isopropanol (m.p. 164–166°). Molecular formula: $C_{19}H_{12}N_4O_4$.

The u.v. spectrophotometric characteristics as well as the i.r. spectra of both Metabolite I and Nitrimidazine are reported in Table 2 and Fig. 1 respectively:

TABLE 2. U.V. SPECTRA OF NITRIMIDAZINE AND METABOLITE I

Spectrophotometric characteristics	λ_{max} in MetOH	E_1^1 λ_{max} in MetOH	λ_{max} H_2SO_4 2N	E_1^1 λ_{max} H_2SO_4 2N
Nitrimidazine	299	310	270	248
Metabolite I	299	325	267	250

The investigation on u.v. spectra has allowed us to conclude that the metabolic conversion that has lead to the formation of compound I has not involved the cycle of nitroimidazole; on the other hand, it can be deduced from the i.r. spectrum that the presence of a $-C=O$ group in the metabolite represents the essential structural difference between Nitrimidazine and the said metabolite. With regard to the position of this group, it can be said that the frequency rate of its stretching vibration (1642 cm^{-1}) allows to exclude the α position (Fig. 8).

On the other hand, the β position results to be excluded by the simple comparison of the physicochemical characteristics of an actual sample having the $-C=O$ group in the β position with that of the metabolite isolated from the urine. It can therefore be concluded that the 1-(*N*-β-ethyl-3-oxomorpholine)-5-nitroimidazole

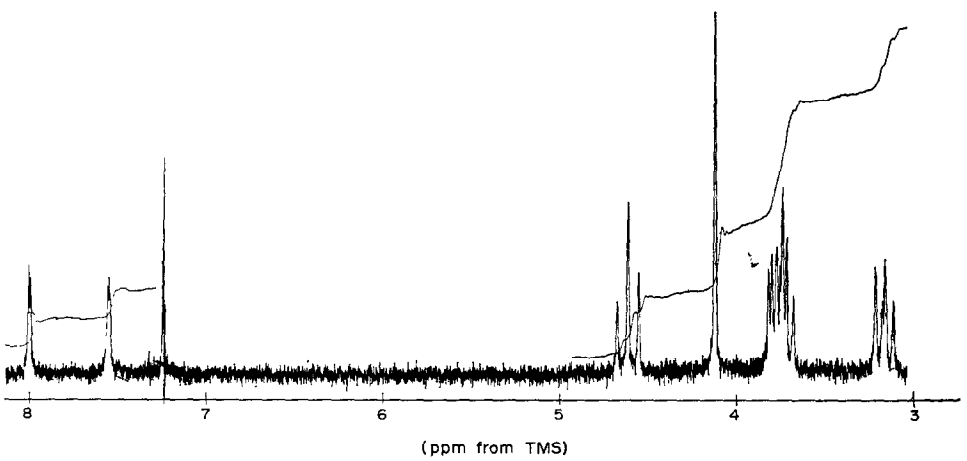


FIG. 2. N.m.r. spectrum in $CDCl_3$ of Metabolite I.

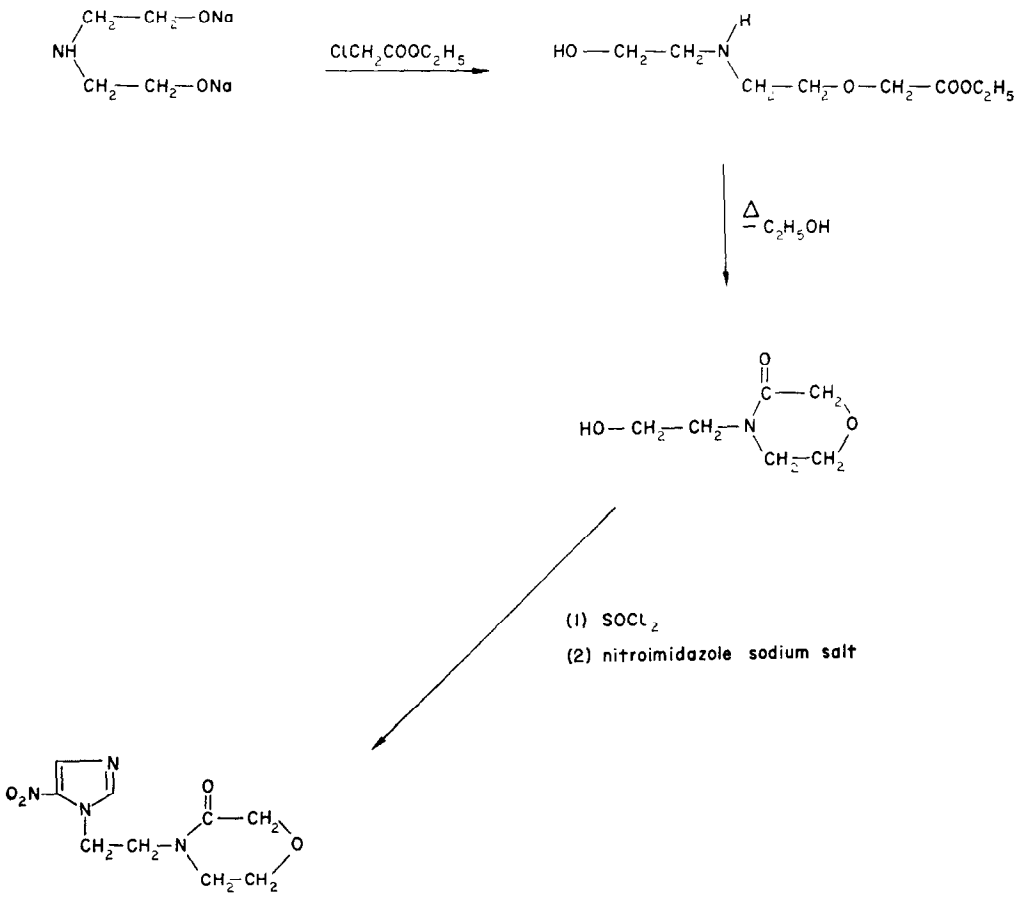
structure can be attributed to the metabolite which can be extracted with CH_2Cl_2 from the urine of human subjects treated with Nitrimidazine. This structure is also suggested by the thorough examination of the n.m.r. spectrum (Fig. 2).

The spectrum of the carbonyl compound is consistent with the proposed structure. A singlet is at very low field as 4·132 because of the electron withdrawing effect of the oxygen and carbonyl group. These two protons are almost equivalent in such a way as to give no observable multiplicity and are attributed to δ' position (Fig. 8).

Bands at 4·62 and 3·74 are parts of a first order pattern A_2X_2 arising from the CH_2–CH_2– side chain methylene protons.

Bands at 3·76 and 3·15 are typical of a higher order system $A_2'X_2'$ and are due to the CH_2–CH_2– of the morpholine ring.

The methylene protons on the ring, closest to the nitrogen of the lactamic group are further downfield because of the electron withdrawing effect of the carbonyl compared with ones of the morpholine ring. The above hypotheses have been confirmed by comparison of the physico-chemical characteristics (m.p.; TLC; i.r.; n.m.r.) of metabolite I with those prepared according to the following scheme:



Chemical. All melting points are uncorrected.

N-(*β-hydroxy-ethyl*)-3-*oxo-morpholine*. To a stirred solution of diethanolamine (60 g) in dioxane (360 ml) metallic sodium (11·4 g) was added in small portions. The mixture was heated cautiously to obtain a temperature of 95° and then refluxed for 4 hr, during which time a white suspension was obtained.

The mixture was cooled, treated with ethyl chloroacetate (57 ml) and then refluxed for 6 hr. The cooled suspension was filtered and the solid washed with hot ethanol (150 ml). The filtrate was evaporated to dryness in vacuo and the residue distilled under reduced pressure to give *N*-(β-hydroxy-ethyl)-3-oxo-morpholine (57·6) g, b.p. 176°/1·5 mm.

(Found: N, 9·65; O, 32·92; $C_6H_{11}NO_3$ requires: N, 9·64; O, 33·07.)

N-(*β-chloro-ethyl*)-3-*oxo-morpholine*. To a stirred solution of *N*-(β-hydroxy-ethyl)-3-oxo-morpholine (50 g) in dry benzene (250 ml) was added dry pyridine (47 ml) and then dropwise, a solution of thionyl chloride (30·5 ml) in dry benzene (35 ml) at room temperature. The mixture was refluxed for 2 hr and then evaporated to dryness in vacuo; the residue was dissolved in methylene chloride (200 ml). The solution was washed with water (4 × 20 ml) and dried over magnesium sulfate. The methylene chloride was removed *in vacuo* and the residue distilled under reduced pressure to give *N*-(β-chloro-ethyl)-3-oxo-morpholine (39·5g), b.p. 118°/1 mm.

(Found: N, 8·43; Cl, 21·70; $C_6H_{10}ClNO_2$ requires: N, 8·56; Cl, 21·66.)

1-(N-*β-ethyl*-3-*oxo-morpholine*)-5-*nitro-imidazole*. To a suspension of sodium salt of 4(5)-nitro-imidazole (13·5 g) in dry dioxane (160 ml) was added dropwise with stirring, at room temperature, a solution of *N*-(β-chloro-ethyl)-3-oxo-morpholine (20·2 g) in dry dioxane (40 ml). The mixture was refluxed for 3 hr and then cooled to 20°.

The theoretical amount of sodium chloride was filtered; the solvent was removed under reduced pressure and the resultant oil solidified by petroleum ether (b.p. 40–70°) addition, obtaining a mixture of two isomers 4 and 5 nitro. For the separation of two isomers the mixture (21·6 g) was suspended in ethanol (125 ml), treated with ethanolic hydrogen chloride (30%) (20 ml) and stirred at room temperature for 1 hr, then refluxed for ½ hr, during which time an amount of ethanol was added for dissolving the suspension. The solution was decolorized, filtered and cooled to 0° obtaining the 1-(*N*-β-ethyl-3-oxo-morpholine)-5-nitro-imidazole HCl, m.p. 184–186°. The mother liquors contain the 4-nitro-isomer.

A solution of 1-(*N*-β-ethyl-3-oxo-morpholine)-5-nitro-imidazole HCl (7·5 g) in water (50 ml), heated at 50°, was neutralized with sodium hydroxide solution (8%) and then stirred at 50° for 1 hr.

The mixture was cooled to 15°, the product was collected by filtration and washed well with cold water giving 1-(*N*-β-ethyl-3-oxo-morpholine)-5-nitro-imidazole, m.p. 164–166°.

(Found: C, 44·72; H, 5·10; N, 23·20; O, 26·91. $C_9H_{12}N_4O_4$ requires: C, 44·99; H, 5·03; N, 23·32; O, 26·64.)

(b) *Metabolite II*

The urine of human subjects treated orally with 500 mg of Nitrimidazine is extracted with a mixture of chloroform–ethanol (3:2). The organic phase is evaporated to dryness, dissolved in methylene chloride and extracted with water.

The aqueous solution is acidified with HCl pH 1–3 and washed twice with a half

volume of ethyl acetate. It is then adjusted at pH 6–7, concentrated to half volume and added with an equal volume of a 2% solution of hot picric acid. The resulting solution is allowed to cool and the precipitation of picrate is obtained. The picrate, dissolved in methanol, is transferred on a Dowex ion exchange resin in the form of OH^-. The eluate is concentrated to dryness and crystallized from acetone.

Metabolite II is thus isolated as a pale yellow powder (m.p. 135–136°).

Molecular formula: $C_9H_{14}N_4O_4$.

Table 3 reports the values of $R_f \times 100$ compared with those of Nitrimidazine.

TABLE 3. VALUES OF $R_f \times 100$ OF NITRIMIDAZINE AND METABOLITE II

Running phase	A	B	C	D
Nitrimidazine	95	95	83	37
Metabolite II	55	60	52	9·5

A = $CHCl_3$–CH_3OH–$(C_2H_5)NH$ (70:20:10)
B = $CHCl_3$–CH_3OH–NH_4OH (70:20:5)
C = C_3H_7OH–NH_4OH (190:10)
D = CH_3COCH_3–CH_3COOH (180:20).

Metabolite II, in aqueous solution at pH 1·5 with sodium metabisulfite, is quantitatively converted into Nitrimidazine.

Figures 3 and 4 show the i.r. spectra of Nitrimidazine and Metabolite II respectively.

When the spectra of the two compounds are compared, it can be observed that Metabolite II shows the disappearance of the medium intensity absorbance at 2800 and 2820 cm^{-1} present in Nitrimidazine. These findings lead us to assume the disappearance of the free electron pair on the morpholine nitrogen, which would produce a higher intensity for the aliphatic C–H stretchings.[9–11] Moreover the absorbance

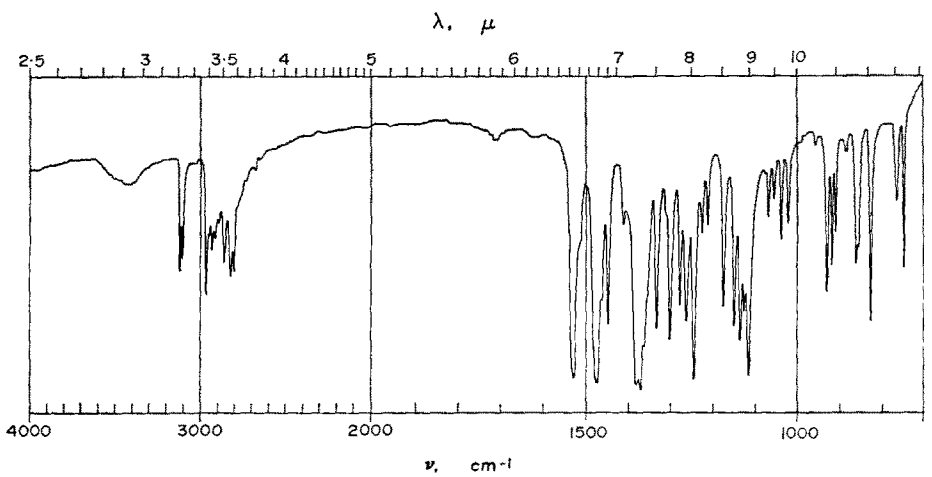


FIG. 3. Infrared spectrum in KBr of nitrimidazine.

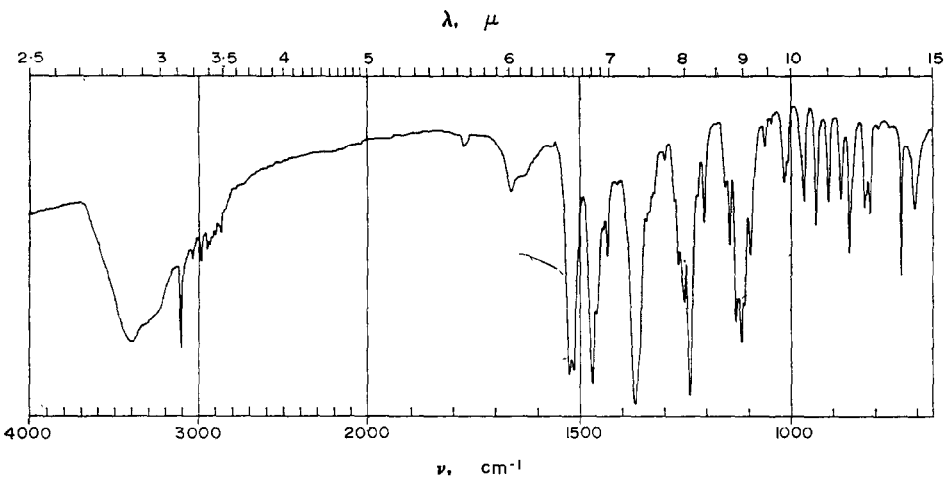


FIG. 4. Infrared spectrum in KBr of Metabolite II.

ranging between 900 and 1000 cm^{-1} are markedly unlike those of Nitrimidazine. The appearance of a medium intensity absorbance at 965 cm^{-1} might represent the N–O stretching for the aliphatic *N*-oxides.[12] These data allow us to assume that the compound may actually be Nitrimidazine-*N*-oxide.

Following, in fact, the treatment of Nitrimidazine with H_2O_2 in CH_3COOH, a product is obtained whose physicochemical characteristics are identical to those of the product separated from the urine.

Figure 5 shows the n.m.r. spectrum of Metabolite II, and also demonstrates the *N*-oxide position of the nitrogen atom of the morpholine ring.

The n.m.r. spectrum of *N*-oxide (solvent $CDCl_3$) when compared with the spectrum

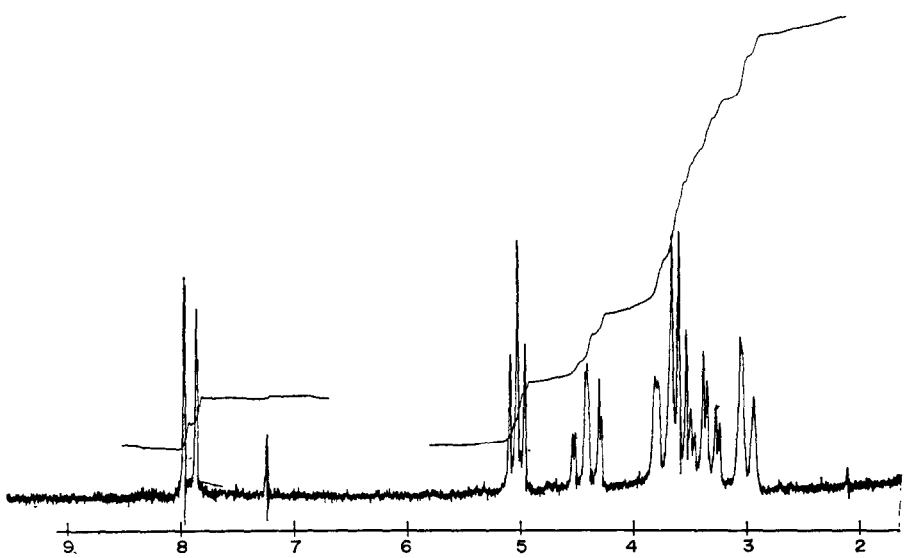


FIG. 5. N.m.r. spectrum in $CDCl_3$ of Metabolite II.

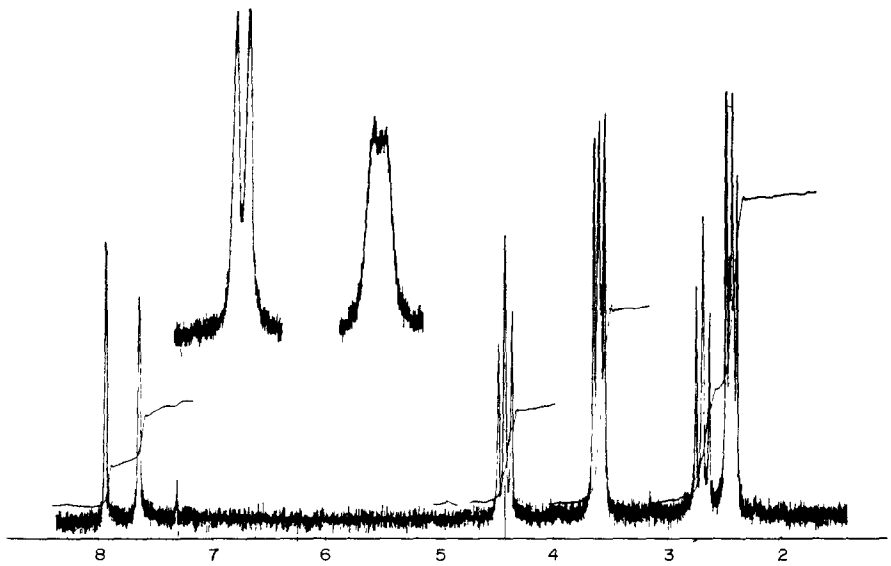


FIG. 6. N.m.r. spectrum in $CDCl_3$ of nitrimidazine (PPM from TMS).

of the Nitrimidazine (Fig. 6) shows the disappearance of the symmetric pattern $A_2'X_2'$ due to the flexible morpholine ring.

It is therefore likely that molecular change due to the *N*-oxide function in the immediate environment of the ring protons causes the observed lack of symmetry. Only a complete analysis of this high order system (probably $A_2B_2\ X_2K_2$) can explain the whole spectrum with the probably more stable axial *N*-oxide.

Chemical. 1-(N-*β-ethyl-morpholine*-N-*oxide*)-5-*nitro-imidazole.* To a solution of 1-(*N*-*β*-ethyl-morpholine)-5-nitro-imidazole (10 g) in acetic acid (50 ml) was added hydrogen peroxide (30%) (25 ml).

The solution was allowed to stand at room temperature for 60 hr.

The solution was then evaporated to dryness and the residue, dissolved in water, was neutralized with sodium hydroxide solution. The mixture was evaporated, extracted with dry methylene chloride; the organic layer was dried over anhydrous sodium sulfate and evaporated to dryness *in vacuo*. The residue was crystallized from acetone to give 1-(*N*-*β*-ethyl-morpholine-*N*-oxide)-5-nitro-imidazole, m.p. 135–136°. (Found: C, 44·85; H, 5·98; N, 23·28; O, 26·05. $C_9H_{14}N_4O_4$ requires: C, 44·62; H, 5·82; N, 23·13; O, 26·42.)

(c) *Quantitative determination of urinary metabolites*

Figure 7 shows the distribution coefficient of Nitrimidazine and of the two metabolites between CH_2Cl_2 and buffers at various pH.

As can be seen, Metabolite I is extracted at an acidic pH while Nitrimidazine can only be extracted at an alkaline pH.

Metabolite II cannot be extracted immediately but has to be previously reduced to Nitrimidazine with $Na_2S_2O_5$.

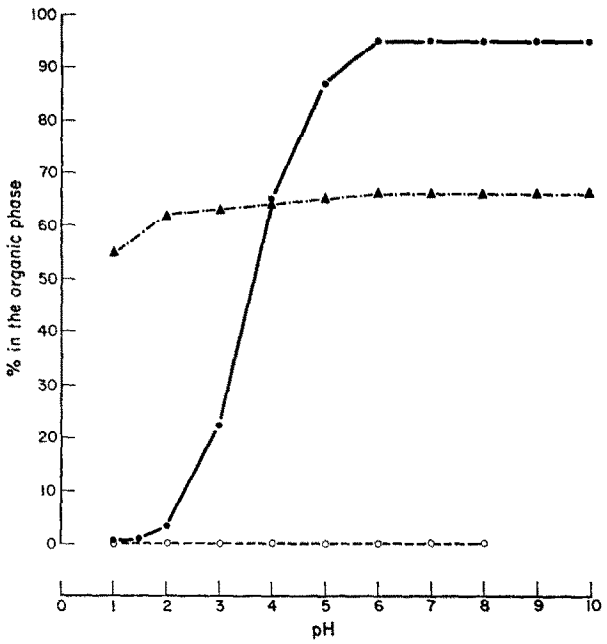


FIG. 7. Distribution coefficient of nitrimidazine ●—●. Metabolite I ▲—▲ and Metabolite II ○—○ between CH_2Cl_2 and buffers.

The urine is acidified at pH 1 with 2N HCl, and extracted with methylene chloride. The organic phase is washed with 0·1 N NaOH, then with water, evaporated and gas-chromatographed on a 2 m-long column, 0·6% C.H.M.D.S. stationary phase on Gas Chrom. P. 100–120. In this way Metabolite I is quantitatively determined. The residual aqueous phase, containing both Metabolite II and Nitrimidazine, is basified to pH 9–10 with 2N NaOH, extracted with CH_2Cl_2, evaporated and gas-chromatographed according to the above described procedure and thus Nitrimidazine is quantitatively determined.

To determine Metabolite II a sample of urine, after extraction with CH_2Cl_2 which extracts both Nitrimidazine and Metabolite I, is acidified to pH 1, treated with $Na_2S_2O_5$, basified to pH 9–10 with 2N NaOH, extracted with CH_2Cl_2, and treated according to the above described procedure.

Consequently, Metabolite II is determined after its conversion into Nitrimidazine. The working temperature of the gas-chromatograph column is 200°, the retention time is 7 min 20 sec for Nitrimidazine; the equivalent values are 240° and 6 min 20 sec for Metabolite I.

Further details on the gas-chromatographic procedure concerning this determination are now in the press.

RESULTS AND DISCUSSION

On the basis of what has been described above with regard to the isolation and the identification of urinary metabolites, the metabolic conversion of Nitrimidazine in humans can be schematized as follows:

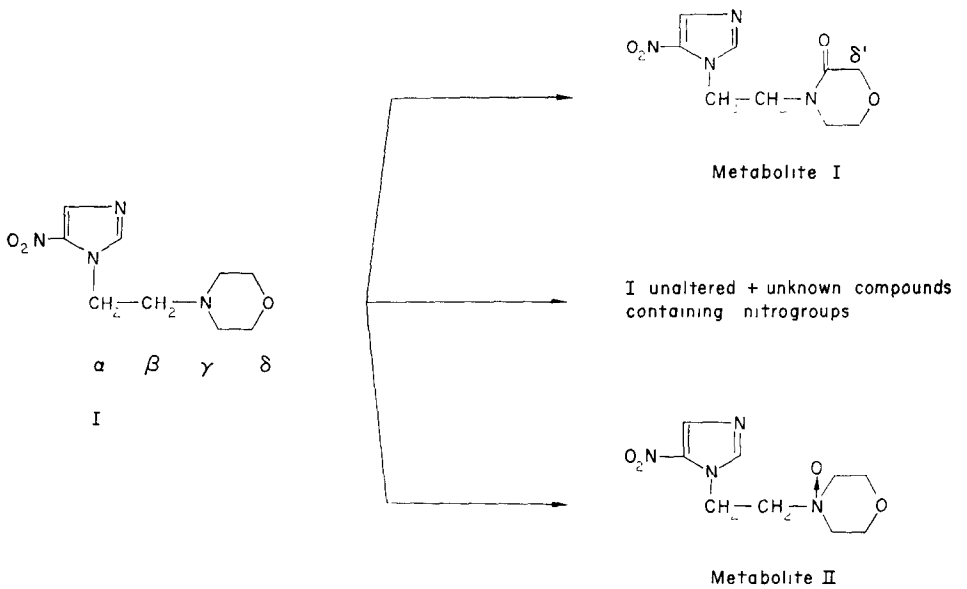


FIG. 8. Scheme of the metabolisms in humans of 1-(*N*-β-ethylmorpholine)-5-nitro-imidazole.

The quantitative determination has been carried out, with the gas-chromatographic technique, on the urine of a human subject treated with a 500 mg-dose of Nitrimidazine.

The urine, collected for a period of 48 hr, has provided the following results:

Unaltered Nitrimidazine	1·23%
Metabolite I	9·02%
Metabolite II	24·57%

Total amount eliminated in the urine, compared with dosage given 34·82% (mean of four subjects).

The urine still contains a 20% aliquot of compounds containing nitrogroups, determined polarographically, on the nature of which we are at the moment investigating.

To the purpose of gaining a better perspective of the antiprotozoan activity of this metabolic pattern, we are herewith reporting in comparison with Nitrimidazine the *in vitro* trichomonacidal activity of the two metabolites on the M strain of *Trichomonas vaginalis*.

Nitrimidazine	0·3 mcg/ml
Metabolite I	1·4 mcg/ml
Metabolite II	0·6 mcg/ml

It is interesting to point out the high activity shown by the two metabolites of Nitrimidazine.

This finding represents, in our opinion, a very interesting aspect revealed by the metabolic investigations carried out on this substance.

Acknowledgement—The authors express their appreciation to Dr. E. Pella for microanalyses, C. E. Confalonieri for infrared spectra, E. Moro for gas-chromatographic determination and to F. Casabuona and G. Longo for technical assistance.

REFERENCES

1. P. N. Giraldi, V. Mariotti and I. de Carneri, *J. med. chem.* **11**, 66 (1968).
2. P. N. Giraldi, V. Mariotti, G. Nannini, G. P. Tosolini, E. Dradi, W. Logemann, I. de Carneri and G. Monti, *Arzneimittelforsch.* **20**, 52 (1970).
3. I. de Carneri, A. Cantone, A. Emanueli, P. N. Giraldi, W. Logemann, R. Longo, G. Meinardi G. Monti, G. Nannini, G. P. Tosolini and G. Vita, *6th Intern. Congr. Chemother.*, Tokyo (1969).
4. A. Cantone, I. de Carneri, A. Emanueli, P. N. Giraldi, W. Logemann, R. Longo, G. Meinardi, G. Monti, G. Nannini, G. Tosolini and G. Vita, *Mal. Infett.* **21**, 954 (1969).
5. I. Signorelli and A. Emanueli, *Minerva gin.* **21**, 1649 (1969).
6. V. Rognoni and L. Sideri, *Rivista Ostet. Ginec. prat.* **51**, 237 (1969).
7. M. Ricci, *Minerva gin.* in press.
8. A. Emanueli and F. Bianchi, *Riv. Ginec. prat.* in press.
9. F. Bohlmann, *Chem. Ber.* **91**, 2157 (1958).
10. J. B. Lambert, *J. Am. chem. Soc.* **89**, 3761 (1967).
11. J. Dabrowski, *Bull. Acad. Pol. Sci.* **15**, 587 (1967).
12. L. J. Bellamy, *The Infrared Spectra of Complex Molecules* 308 (1958).