CHROM. 5286

Determination of the urinary levels of a new trichomonacidal agent and its main metabolites by gas–liquid chromatography

In the course of investigations involving nitroimidazole derivatives with antiprotozoan activity[1,2], 1-(N-β-ethylmorpholine)-5-nitroimidazole (Nitrimidazine*) was found to exhibit particularly good trichomonacidal activity both *in vitro* and *in vivo*[3,4]; moreover this compound proved to be highly active in human giardiasis, intestinal and hepatic amebiasis. From work[5] dealing with the isolation and identification of urinary metabolites of 1-(N-β-ethylmorpholine)-5-nitroimidazole, it appears that when humans are treated with 1-(N-β-ethylmorpholine)-5-nitroimidazole, in addition to the said product, two of its principal metabolites, namely 1-(N-β-ethyl-3-oxomorpholine)-5-nitroimidazole and 1-(N-β-ethylmorpholine-N-oxide)-5-nitroimidazole, occur in urine; their structures, as determined by their IR and NMR spectra, are shown in Fig. 1. In order to estimate the amount of 1-(N-β-ethylmorpholine)-5-nitroimidazole excreted unchanged in the urine of human subjects treated with it and the amount of the two metabolites mentioned above, we developed a specific procedure for the extraction of these compounds and a method, based on GLC, for their quantitative determination.

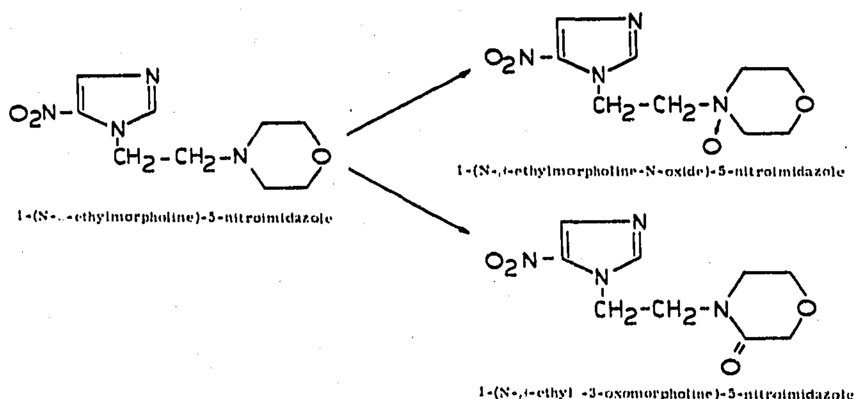


Fig. 1. Structures of 1-(N-β-ethylmorpholine)-5-nitroimidazole and its main metabolites present in human urine.

Materials and methods

Substances and abbreviations. Throughout this paper, 1-(N-β-ethylmorpholine)-5-nitroimidazole, 1-(N-β-ethyl-3-oxomorpholine)-5-nitroimidazole and 1-(N-β-ethylmorpholine-N-oxide)-5-nitroimidazole will be designated I, II and III, respectively; they were synthesized by methods described in literature[2,5]. All three products yielded a single spot in TLC on silica gel; the R_F values of I and II are 0.32 and 0.12, respectively, using benzene–acetone–diethylamine (170:20:10) as solvent system; the R_F of III is 0.52 using *n*-propanol–ammonia (32%) (190:10) as solvent system. The detection of the chromatographic spots was effected by visualisation under UV light (254 mμ) and by reduction with titanous chloride followed by diazotization with amyl nitrite and coupling with N-(1-naphthyl)-ethylenediamine. The titer of the three sub-

* Proprietary name: Naxogin (Carlo Erba S.p.A. Milan, Italy).

stances (potentiometric titration with $HClO_4$ in nitroethane) was 100 % for I and II, and 99.4 % for III. 4-Hydroxydiphenyl (4-OH-DP) and cholesteryl acetate (CHOL-AC) used as internal standards for quantitation in the GLC determinations were obtained from Carlo Erba S.p.A. and B.D.H. Italia S.p.A., respectively, and required no further purification. All other solvents and reagents were Carlo Erba R.S.

Partition coefficients. Since the most effective solvent for the extraction of I and II from aqueous solutions was found to be CH_2Cl_2, we determined the variation of the partition coefficients of the said substances between CH_2Cl_2 and aqueous buffers, as a function of the pH of the latter. To this end the two phases were mutually saturated before the partition experiment; then the substances under study were dissolved in a suitable volume of the aqueous phase which was shaken for 12 h with the organic solvent; lastly the two phases were left to separate overnight. The whole procedure was carried out at 25 $\pm$ 2°. The initial concentration of the substances in the aqueous phase and their concentration in the same phase after partition were determined spectrophotometrically. The partition procedure was also carried out with III, which is much more water-soluble than the other two compounds. Fig. 2 shows the percentages (averages of three determinations) of I, II and III extracted by the organic solvent as calculated from their partition coefficients.

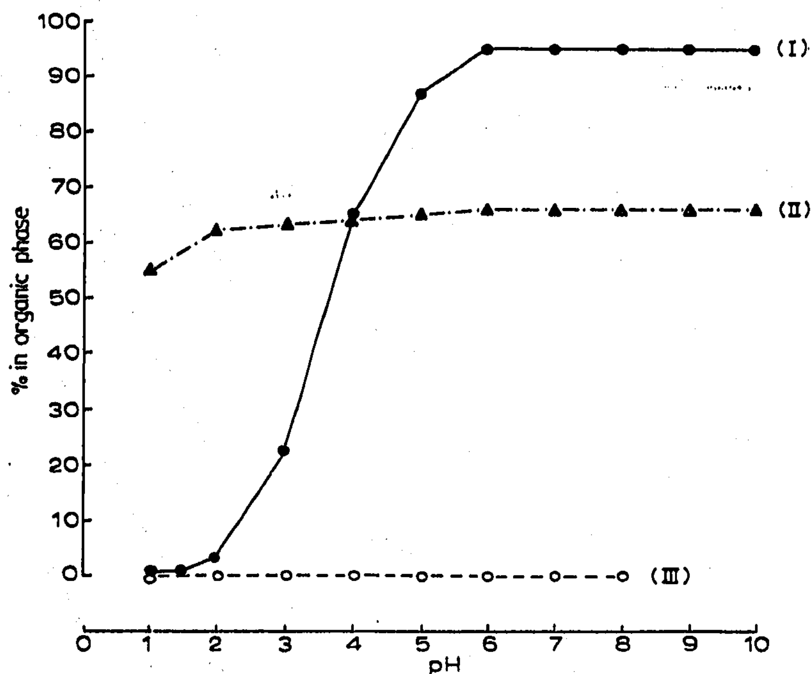


Fig. 2. Partition of 1-(N-β-ethylmorpholine)-5-nitroimidazole (I), 1-(N-β-ethyl-3-oxomorpholine)-5-nitroimidazole (II) and 1-(N-β-ethylmorpholine-N-oxide)-5-nitroimidazole (III) between aqueous buffers and CH_2Cl_2 *vs.* pH of the buffers.

Extraction procedure. From the data shown in Fig. 2 it is apparent that the most convenient pH range of the aqueous solution for the extraction of I lies between 4 and 10 and that this compound cannot be extracted by CH_2Cl_2 from aqueous solutions at pH's near 1. Furthermore the same data show that II is easily extracted by the organic solvent over the whole pH range examined while III cannot be extracted from the aqueous solution at any of the pH values taken into consideration. For this last

reason we sought a reaction by which the N-oxide group of III could be selectively reduced and, consequently, III transformed into I. On the other hand, all reducing agents currently used to de-oxidize N-oxides in aqueous solutions (for example, $TiCl_3$), in addition to the desired reaction, also reduce to a variable degree the nitro group at position 5 in the imidazole ring of III. The only reagent, among those tested, which carries out the de-oxidation without side reactions was found to be $Na_2S_2O_5$ in an acidic medium (pH = 1–1.5); under these conditions, both in aqueous solutions and in urine, the N-oxide group is reduced quantitatively within 15 min at room temperature and III is converted to I. Furthermore, TLC confirms that this treatment does not cause any other transformation in the structure of III. On the basis of the findings reported above, we found that the procedures described in the following paragraphs afford effective extraction of I, II and III from urine, yielding extracts that are free from interference when subjected to GLC.

Extraction of I and II. From a 10 ml sample of urine, adjusted to pH 1 (controlled by pH meter) with 2 *N* hydrochloric acid, II is extracted for 15 min by mechanical shaking with two 40 ml portions of CH_2Cl_2. While the residual aqueous phase is set aside for the subsequent extraction of I, the combined CH_2Cl_2 extracts are washed with 0.1 *N* sodium hydroxide (2 × 4 ml) and distilled water (2 × 4 ml), then evaporated to dryness under a stream of nitrogen. The residue is redissolved with 0.2 ml of a solution containing 1 mg of CHOL-AC per ml of CH_2Cl_2: a suitable aliquot of the solution thus obtained is submitted to GLC for the determination of II.

For the extraction of I, 5 ml of the residual aqueous phase (set aside from above) is brought to pH 8–9 with 2 *N* sodium hydroxide and extracted for 15 min by mechanical shaking with two 20 ml portions of CH_2Cl_2; the combined organic extracts are washed with distilled water (2 × 2 ml) and evaporated to dryness under a stream of nitrogen. The residue is redissolved with 0.2 ml of a solution containing 0.25 mg of 4-OH-DP per ml of CH_2Cl_2: a suitable aliquot of the solution thus obtained is sub-

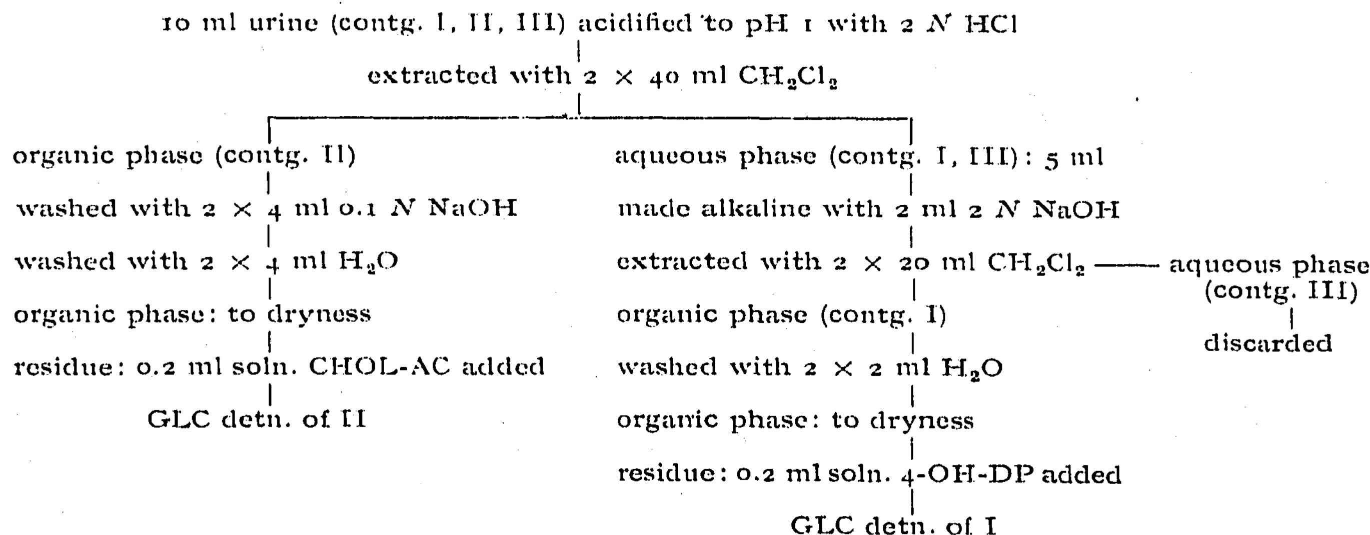


Fig. 3. Scheme for the extraction of 1-(N-β-ethylmorpholine)-5-nitroimidazole (I) and 1-(N-β-ethyl-3-oxomorpholine)-5-nitroimidazole (II) present in urine also containing 1-(N-β-ethylmorpholine-N-oxide)-5-nitroimidazole (III). CHOL-AC = cholesteryl acetate 1 mg/ml CH_2Cl_2. 4-OH-DP = 4-hydroxydiphenyl 0.25 mg/ml CH_2Cl_2.

mitted to GLC for the determination of I. The details of the operations described in this paragraph are summarized in the scheme reported in Fig. 3.

Extraction of III. A 10 ml sample of urine is freed of I and II by extraction with two

10 ml urine (contg. I, II, III)
|
extracted with 2 × 20 ml CH_2Cl_2 —————— organic phase (contg. I, II)
| |
aqueous phase (contg. III): 5 ml   discarded
|
acidified to pH 1–1.5 with 1 *N* HCl
|
25 mg $Na_2S_2O_5$ added
|
after 15 min: made alkaline to pH 8–9 with 1 *N* NaOH
|
extracted with 2 × 20 ml CH_2Cl_2 —————— aqueous phase
| |
organic phase (contg. I from III)   discarded
|
washed with 2 × 2 ml H_2O
|
organic phase: to dryness
|
residue: 0.2 ml soln. 4-OH-DP added
|
GLC detn. of I from III

Fig. 4. Scheme for the extraction of 1-(N-β-ethylmorpholine-N-oxide)-5-nitroimidazole (III) present in urine also containing 1-(N-β-ethylmorpholine)-5-nitroimidazole (I) and 1-(N-β-ethyl-3-oxomorpholine)-5-nitroimidazole (II). 4-OH-DP = 4-hydroxydiphenyl 0.25 mg/ml CH_2Cl_2.

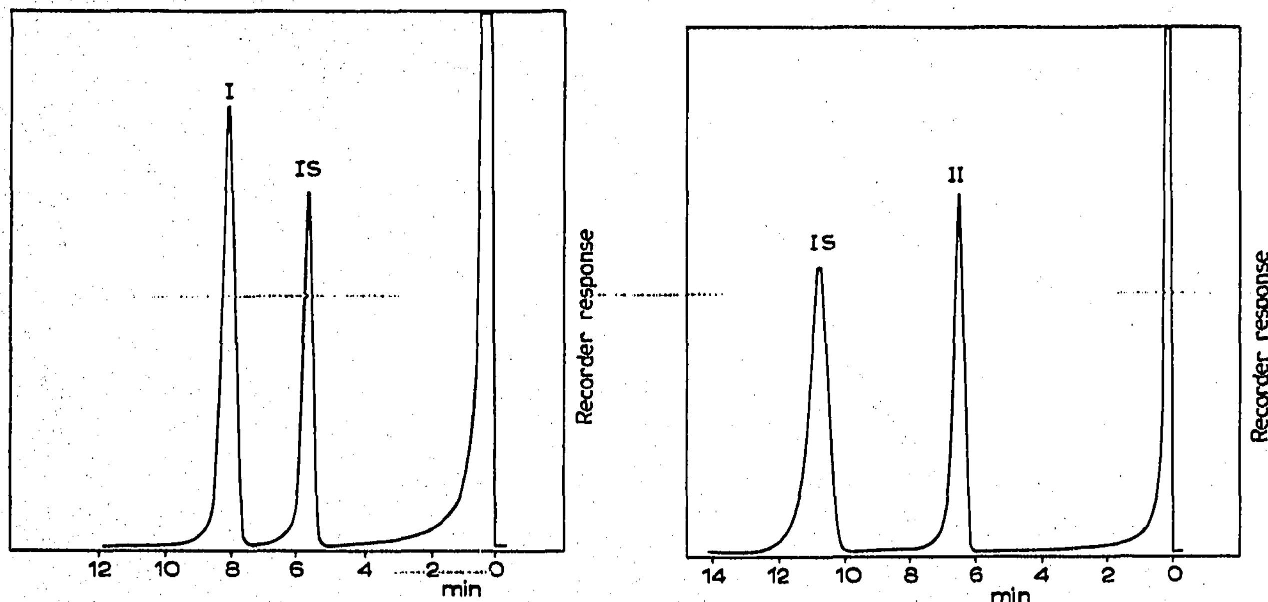


Fig. 5. Gas chromatogram of standard 1-(N-β-ethylmorpholine)-5-nitroimidazole (I) on a 2 m glass column packed with 0.6% CHDMS on Gas-Chrom P (100–120). Column temperature, 200°; carrier gas (N_2) flow rate, 60 ml/min; chart speed, 0.5 in./min. Peaks: I = 1-(N-β-ethylmorpholine)-5-nitroimidazole; IS = internal standard (4-hydroxydiphenyl).

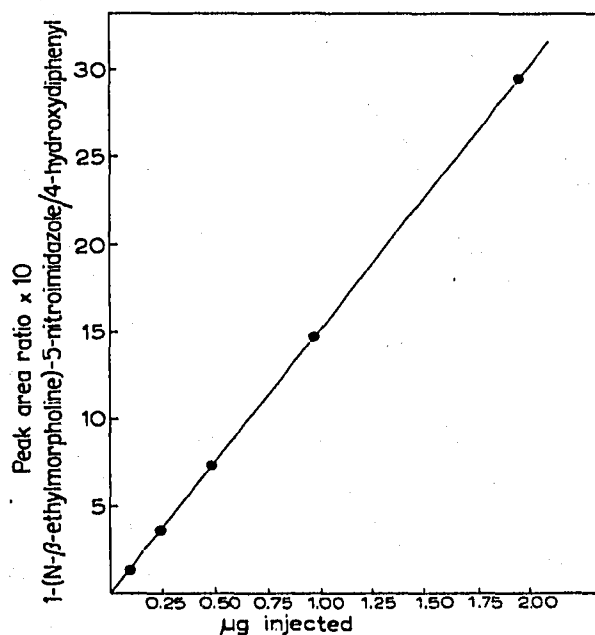
Fig. 6. Gas chromatogram of standard 1-(N-β-ethyl-3-oxomorpholine)-5-nitroimidazole (II) on a 2 m glass column packed with 0.6% CHDMS on Gas-Chrom P (100–120). Column temperature, 240°; carrier gas (N_2) flow rate, 60 ml/min; chart speed, 0.5 in./min. Peaks: II = 1-(N-β-ethyl-3-oxomorpholine)-5-nitroimidazole; IS = internal standard (cholesteryl acetate).

20 ml portions of CH_2Cl_2; 5 ml of the residual aqueous phase (now containing only III) are adjusted to pH 1–1.5 (controlled by pH meter) with 1 N hydrochloric acid, and submitted to reduction by addition of 25 mg of $Na_2S_2O_5$; after 15 min, the amount of I obtained by de-oxydation of III is extracted from the aqueous phase (after adjusting to pH 8–9 with 1 N sodium hydroxide) following the steps described above for the extraction of I. The details of the operations described in this paragraph are summarized in the scheme reported in Fig. 4.

Gas–liquid chromatography. The instrument employed in this investigation was a Carlo Erba model G.V. equipped with flame ionization detector (H_2 = 35 ml/min, air = 400 ml/min). A U-shaped glass column (length 2 m, I.D. 4 mm) packed with 0.6% CHDMS on acid washed silanized 100–120 mesh Gas-Chrom P was used; coating of the support was effected by the filtration technique. Nitrogen was used as carrier gas. In the determination of I the temperatures were as follows: column 200°, injector port 230°, detector 240°; in the determination of II the temperatures of the column, injector port and detector were 240, 260 and 280° respectively; in both cases the carrier gas flow rate was 60 ml/min.

Quantitative evaluation. The area of the chromatographic peaks was calculated by multiplying the peak height by the width of the peak at half its height. Quantitative evaluations were made (as stated above) with reference to the area of internal standards consisting of 4-OH-DP at a concentration of 0.25 mg/ml CH_2Cl_2 in the determination of I and of CHOL-AC at a concentration of 1 mg/ml CH_2Cl_2 in the determination of II. Under the GLC working conditions specified above, the retention times of the test substances and internal standards are as follows: I = 8 min; II = 6 min 20 sec; 4-OH-DP = 5 min 40 sec; CHOL-AC = 10 min 40 sec (Figs. 5 and 6).

Calibration curves. Figs. 7 and 8 show the calibration curves for I and II. In both



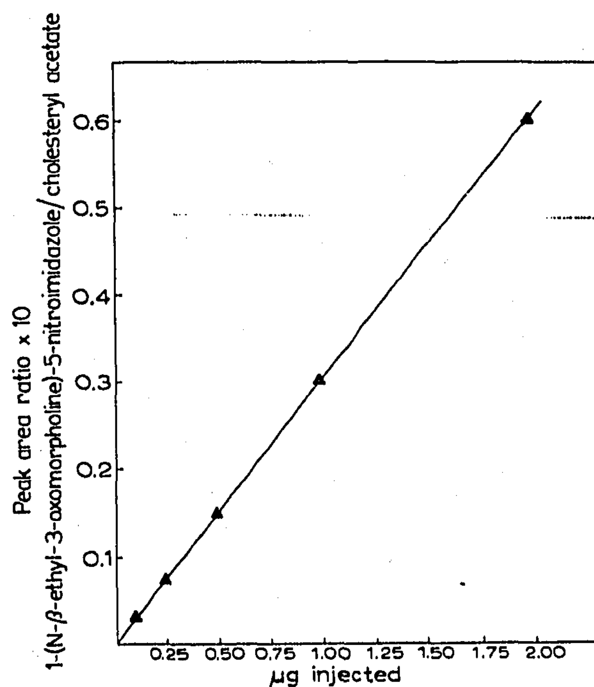


Fig. 7. Calibration curve for 1-(N-β-ethylmorpholine)-5-nitroimidazole (I).

Fig. 8. Calibration curve for 1-(N-β-ethyl-3-oxomorpholine)-5-nitroimidazole (II).

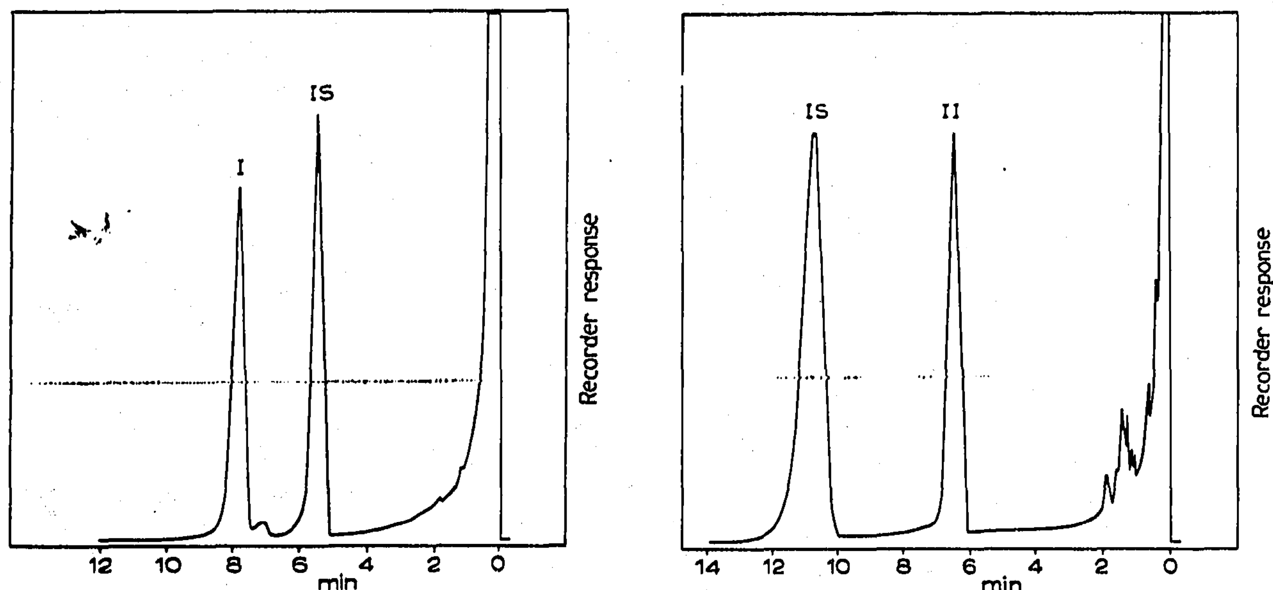


Fig. 9. Gas chromatogram of the urinary extract of a human subject treated with 500 mg of 1-(N-β ethylmorpholine)-5-nitroimidazole (I). Injection equivalent to 1/5000th of the 0–48 h urine after treatment. Peaks: I = 1-(N-β-ethylmorpholine)-5-nitroimidazole; IS = internal standard (4-hydroxydiphenyl). Same conditions as Fig. 5.

Fig. 10. Gas chromatogram of the urinary extract of a human subject treated with 500 mg of 1-(N-β-ethylmorpholine)-5-nitroimidazole (I). Injection equivalent to 1/50000th of the 0–48 h urine after treatment. Peaks: II = 1-(N-β-ethyl-3-oxomorpholine)-5-nitroimidazole; IS = internal standard (cholesteryl acetate). Same conditions as Fig. 6.

TABLE I

REPLICATE DETERMINATIONS AND RECOVERIES OF 1-(N-β-ETHYLMORPHOLINE)-5-NITROIMIDAZOLE (I) FROM URINE

μg of I added to 10 ml urine	*μg of I found in replicate determinations*	*Mean % recovery ± standard deviation*
201.25	162.0; 153.2; 155.1	77.9 2.3
100.62	77.1; 82.5; 77.6	78.6 3.0
50.31	39.5; 36.8; 39.9	76.9 3.3
20.12	16.2; 15.8; 14.6	77.1 4.3

Overall mean % recovery ± standard deviation = 77.6 ± 3.3

TABLE II

REPLICATE DETERMINATIONS AND RECOVERIES OF 1-(N-β-ETHYL-3-OXOMORPHOLINE)-5-NITROIMIDAZOLE (II) FROM URINE

μg of II added to 10 ml urine	*μg of II found in replicate determinations*	*Mean % recovery ± standard deviation*
201.00	148.2; 145.6; 147.4	73.2 0.7
100.50	70.6; 75.4; 77.4	74.1 3.5
50.25	37.1; 37.5; 36.8	73.9 0.7
20.10	15.2; 15.3; 14.0	73.7 3.6

Overall mean % recovery ± standard deviation = 73.7 ± 2.5

TABLE III

REPLICATE DETERMINATIONS AND RECOVERIES OF 1-(N-β-ETHYLMORPHOLINE-N-OXIDE)-5-NITROIMIDAZOLE (III) FROM URINE

μg of III added to 10 ml urine	*μg of III found in replicate determinations*	*Mean % recovery ± standard deviation*
200.62	146.4; 154.1; 143.0	73.7 2.9
100.31	74.1; 71.1; 71.9	72.1 1.6
50.15	34.7; 35.8; 37.3	71.6 2.6
20.06	14.6; 14.5; 14.9	73.2 1.1
Overall mean % recovery ± standard deviation = 72.7 ± 2.1		

cases we obtained a linear plot over the range 0.125 to 2 μg passing through the origin.

Recoveries. In order to determine the recoveries of I, II and III from urine samples, we added increasing amounts of these substances to 10 ml samples of urine from an untreated subject; we then submitted these samples to replicate determinations of the substances under study following the procedures summarized in the schemes reported in Figs. 3 and 4. The recoveries are shown in Tables I–III.

Results

Figs. 9 and 10 show the chromatograms relative to the determination of I and II in the urine of a subject treated with 500 mg of I; the chromatogram relative to the determination of III is quite similar to that obtained in the determination of I. By submitting the urine of four human subjects treated with 500 mg of I to the procedures described in this paper, we found that the average per cent quantity of I, II and III excreted into the urine during 48 h after administration, relative to the amount administered, are 1.23, 9.02 and 24.57 %, respectively.

Thanks are due to Dr. V. MANDELLI for his valuable advice and to S. COLOMBO and G. LONGO for technical assistance.

Istituto Carlo Erba per Ricerche Terapeutiche, Milan (Italy)

G. P. TOSOLINI
E. MORO
P. N. GIRALDI

1 P. N. GIRALDI, V. MARIOTTI AND I. DE CARNERI, *J. Med. Chem.*, 11 (1968) 66.
2 P. N. GIRALDI, V. MARIOTTI, G. NANNINI, G. TOSOLINI, E. DRADI, W. LOGEMANN, I. DE CARNERI AND G. MONTI, *Arzneimittelforsch.*, 20 (1970) 52.
3 I. DE CARNERI, *Arzneimittelforsch.*, 19 (1969) 382.
4 I. DE CARNERI, A. CANTONE, A. EMANUELI, P. N. GIRALDI, W. LOGEMANN, R. LONGO, G. MEINARDI, G. MONTI, G. NANNINI, G. TOSOLINI AND G. VITA, *6th Int. Congr. Chemother., Tokyo*, 1969.
5 P. N. GIRALDI, G. P. TOSOLINI, E. DRADI, G. NANNINI, R. LONGO, G. MEINARDI, G. MONTI AND I. DE CARNERI, *Biochem. Pharmacol.*, 20 (1971) 339.

Received January 19th, 1971