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Note

Determination of ornidazole and its main metabolites in biological fluids

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Ornidazole (Tiberal) (Fig. 1) is successfully used in the treatment of trichomoniasis, amebiasis and lambliasis. More recently, its efficacy against anaerobic bacteria has assumed increasing significance in clinical practice. In the body, ornidazole is extensively metabolized, with less than 4% of the dose being recovered unchanged in the urine [1,2]. The two major metabolites M1 and M4 (Fig. 1) have revealed *in vitro* antibacterial activity almost equal to that of ornidazole.

A few methods have been published for the determination of ornidazole, including gas chromatography (GC) [3] and high-performance liquid chromatography (HPLC) [4,5]. The GC method for blood involves a derivatization step, and the metabolites are not determined. The HPLC methods are for plasma and blood, but they lack a detailed description of the quantification of the metabolites [4,5]; a disadvantage is the need for a relatively large sample volume [4].

This paper describes an HPLC procedure for the quantification of ornidazole

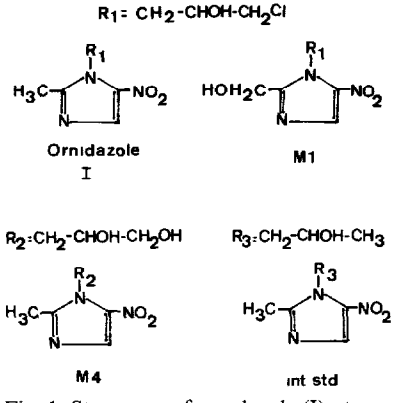

Fig. 1. Structures of ornidazole (I), its metabolites M1 and M4, and the internal standard

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and its main metabolites in plasma, urine, vaginal fluid and cerebrospinal fluid (CSF).

EXPERIMENTAL

Reagents

Ornidazole, its metabolites M1, M4 and the internal standard were from F Hoffmann-La Roche (Basel, Switzerland). Methanol, dichloromethane, $(NH_4)_2SO_4$, Na_2HPO_4 and KH_2PO_4 were p.a. grade from E. Merck (Darmstadt, F.R.G.).

Chromatography

The following modular HPLC system was used: Kontron M 414 HPLC pump (Kontron, Basel, Switzerland), Kratos 773 UV detector (Applied Biosystems, Weiterstadt, F.R.G.), ISS-100 autosampler (Perkin-Elmer, Überlingen, F.R.G.), SP 4100 computing integrator with 4100 D minifile (Spectra Physics, Darmstadt, F.R.G.)

The column (125 mm × 4 mm I.D., Hibar-type, E. Merck) was packed with Nucleosil $5C_{18}$ (Macherey-Nagel, Düren, F.R.G.). The eluent was methanol–0.067 *M* phosphate buffer (pH 6), at a ratio of 80:20 (v/v) for the determination of the parent drug in plasma (CSF) and 17:83 (v/v) for the determination of the metabolites in plasma and for the urine assay. The flow-rate was 1 ml/min, and the monitoring UV wavelength was 312 nm.

Solutions

The following aqueous stock solutions were prepared with the aid of sonication: (a) ornidazole (2 mg/ml); (b) M1 and M4 (1 mg/ml each); (c) ornidazole (1 mg/ml), M1 (0.5 mg/ml) and M2 (1 mg/ml); (d) internal standard (1 mg/ml).

Calibration standards

Stock standards were prepared by adding a suitable amount of the above aqueous solution to drug-free plasm or urine These stock standards were then used to prepare the following lower concentration standards by stepwise dilution with drug-free plasma or urine: plasma standards (parent drug) at 40, 20, 10, 5, 2, 1, 0.5 and 0.25 μg/ml; plasma standards (containing both M1 and M4) at 1000, 500, 250, 100 and 50 ng/ml; urine standards (ornidazole/M1/M4) at 50/25/50, 25/12.5/25, 10/5/10, 5/2.5/5, 2.5/1.25/2.5 and 1.25/0.62/1.25 μg/ml.

The calibration standards were stored frozen in small portions (−20°C).

Sample preparation

Plasma and CSF. Frozen samples were allowed to thaw and warm to room temperature. After vortex-mixing, 0.25 ml of the sample was pipetted into a conical extraction tube (glass, 15 ml). Then 0.1 ml of a suitable aqueous solution

of the internal standard, 0.25 ml of buffer solution (0.5 *M* phosphate buffer, pH 8, for the determination of the parent drug; saturated $(NH_4)_2SO_4$ for metabolite determination) and 6 ml of dichloromethane were added. The samples were extracted on a rotary extractor (10 min at 20 rpm for the determination of ornidazole and 15 min for the determination of M1 and M4) and then centrifuged (5 min, 1000 *g*). The upper aqueous layer was discarded. The organic phase (5 ml) was transferred to another glass tube and evaporated to dryness under nitrogen (water-bath, 35°C). The residue was reconstituted in the mobile phase (500 μl for determination of the parent drug, 250 μl for determination of the metabolites), transferred to a capped glass micro-vial and injected for analysis (20–50 μl for determination of the parent drug and 100 μl for determination of the metabolites).

Urine. A 100-μl volume of the urine sample was mixed with 100 μl of an aqueous solution of the internal standard and 800 μl of saturated $(NH_4)_2SO_4$. The mixture was transferred to an Extrelut-1 extraction column (E. Merck) and allowed to penetrate into the column material. After 10 min, the compounds were extracted three times with 5-ml portions of dichloromethane. The extracts were combined in a conical tube and evaporated to dryness. The residue was redissolved in 500 μl of the mobile phase and then injected for analysis (25–50 μl).

Vaginal fluids. Vaginal fluids (*ca.* 200 mg) were weighed into an extraction tube. After the addition of buffer solution (0.25 ml) and of the internal standard (0.1 ml of a suitable aqueous solution), the procedure was the same as that described for plasma samples. The dry residue was taken up in 500 μl of the mobile phase, and 25–50 μl were injected for analysis.

Calibration

Calibration standards covering the above concentration ranges were processed as described and analysed alongside the unknown samples. A calibration curve was obtained by a weighted $(1/y^2)$ least-squares linear regression of the peak-height ratios *versus* concentration. The regression was then used to calculate the unknown concentrations in the samples. All data-processing and calculations were carried out by the Spectra Physics SP 4100 computing integrator and 4100 D minifile [6]. In the case of CSF and vaginal fluids, plasma standards were used for calibration.

RESULTS

Recovery

Using a single dichloromethane extraction (6 ml), the recovery of the parent drug from plasma and urine was nearly quantitative from pH 4 to 8. The recoveries for M4 and M1 were 25 and 60%, respectively, under these conditions. A second extraction improved the recoveries to 40 and 80%, respectively. A single dichloromethane extraction with saturated $(NH_4)_2SO_4$ buffer improved the re-

TABLE I

INTER-ASSAY PRECISION OF ORNIDAZOLE (I) AND ITS METABOLITES (M4 AND M1)

Calculation from quality control samples, analysed on different days, n = number of replicates

Plasma assay				Urine assay			
Concentration (μ/ml)	Precision (% R.S.D.)			Concentration (μg/ml)	Precision (% R.S.D.)		
	I	M4	M1		I	M4	M1
0.5	6.9 (n=7)			1.25	7.1 (n=16)	5.3 (n=15)	4.2 (n=16)
0.05		8.1 (n=18)	6.2 (n=16)				
5.0	2.0 (n=12)			5.0	3.7 (n=16)	5.9 (n=15)	4.2 (n=16)
0.1		7.7 (n=19)	7.7 (n=19)				
40	2.7 (n=11)			40	6.3 (n=16)	5.8 (n=15)	6.0 (n=16)
1.0		7.9 (n=18)	5.9 (n=19)				

coveries to 45 and 85% for M4 and M1, respectively. Even better recoveries (85% for M4 and 90% for M1) were obtained by liquid–solid extraction on Extrelut-1 cartridges, using saturated $(NH_4)_2SO_4$ as buffer and extracting twice.

Linearity

Linear correlations between peak-height ratios and the concentrations of ornidazole, M1 and M4 were found in the above-mentioned calibration ranges.

Reproducibility

Inter-assay (day-to-day) reproducibility was calculated from spiked samples, which were analysed as replicates on different days, using a new calibration on each day (Table I).

TABLE II

LIMITS OF QUANTIFICATION AND RECOVERIES OF ORNIDAZOLE (I) AND ITS METABOLITES (M4 AND M1)

Compound	Plasma (CSF) assay			Urine assay		
	Limit of quantification		Mean recovery (%)	Limit of quantification		Mean recovery (%)
	μg/ml	% R.S.D		μg/ml	% R.S.D	
I	0.5	6.9	98	0.6	5.5	90
M4	0.05	8.1	45	0.3	4.3	85
M1	0.05	6.2	85	0.3	8.1	90

Limits of quantification

Using the sample volumes decribed above, the limits of quantification were: 0.5 μg/ml (plasma, CSF) and 0.6 μg/ml (urine) for the parent drug; and 0.05 μg/ml (plasma, CSF) and 0.3 μg/ml (urine) for both M4 and M1 (Table II). The limit of quantification for the parent drug in plasma could easily be improved by a factor of 5–10, if necessary.

DISCUSSION

Determination of the metabolites

In plasma, in comparison with the parent drug, very low metabolite concentra-

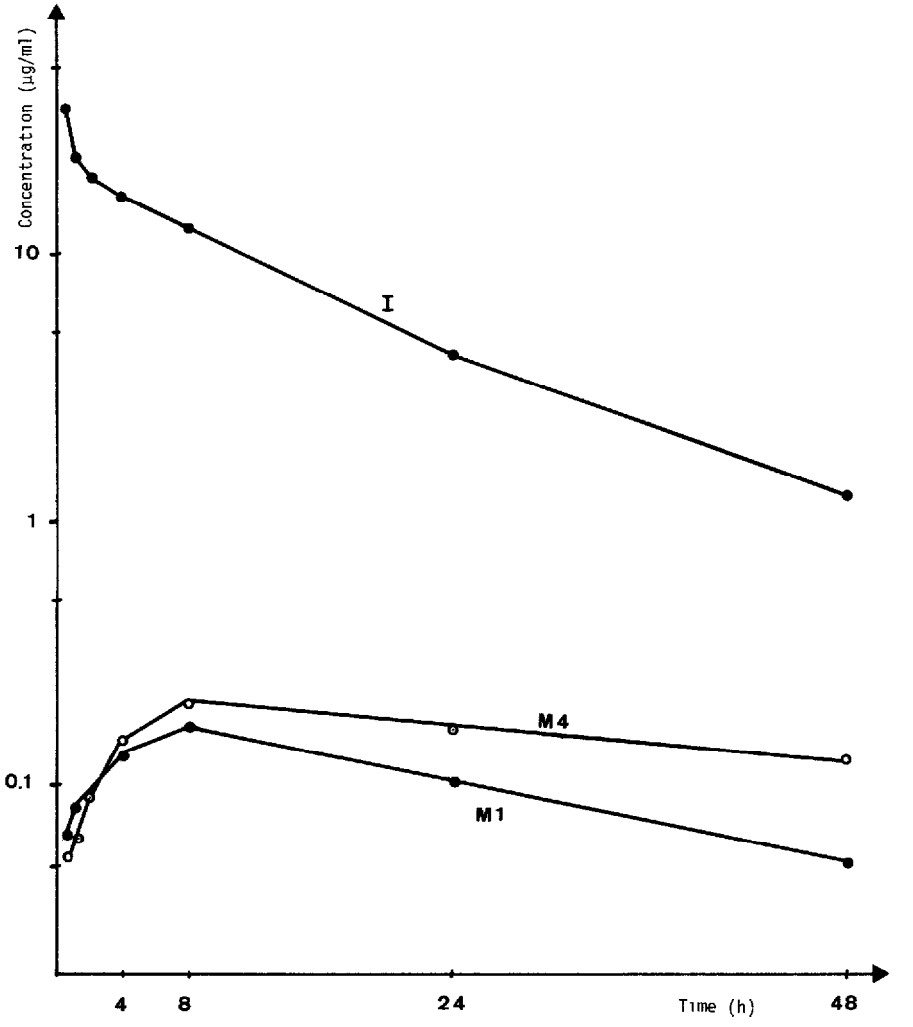

Fig. 2 Plasma levels of ornidazole (I), M4 and M1 following a single 1000-mg infusion of ornidazole.

tions were found (microgram amounts for the parent drug, nanogram amounts for the metabolites, Fig. 2). It was therefore decided to analyse plasma samples in two separate steps, one for the determination of ornidazole and one for the determination of the metabolites. In urine, the parent drug and M1 and M4 could be determined simultaneously.

Extraction of urine samples with Extrelut-1 cartridges improved the recovery for the metabolite M4 and, in addition, resulted in cleaner extracts (Fig. 3). However, this procedure could not be used for plasma samples, because after the addition of saturated $(NH_4)_2SO_4$ to plasma, denatured proteins blocked the extraction cartridge.

Chromatography

Other 5-μm reversed-phase materials than Nucleosil C_{18} could be used with only minor modifications of the eluent composition (*e.g.* LiChrosorb C_{18}, Spherisorb ODS-1). For the determination of the parent drug in plasma, the analysis time may be shortened by using a higher percentage of methanol in the eluent. For the determination of the metabolites, the chromatographic conditions should be sufficiently selective to separate M4 from a small interfering peak that appears in some plasma samples (Fig. 4).

The procedure for the analysis of the metabolites required the injection of large plasma aliquots. Under these circumstances, peak splitting sometimes occurred after the column had been in use for several days. Optimum peak shapes

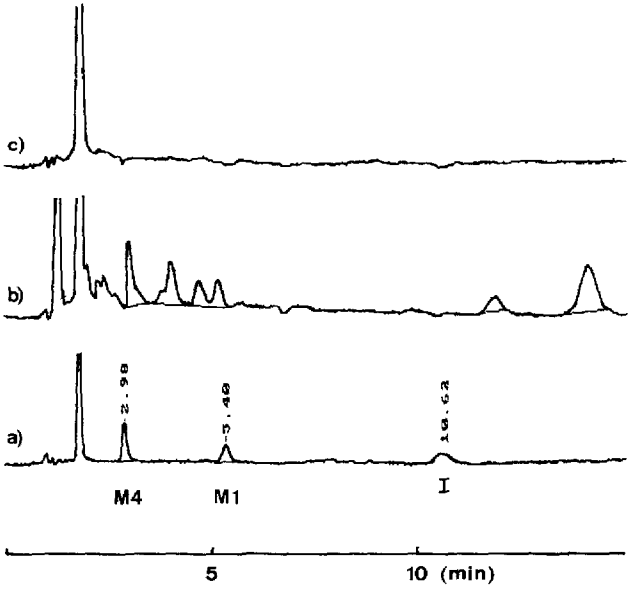

Fig. 3 Effect of Extrelut clean-up procedure. (a) Calibration standard (M4, M1 0.3 μg/ml), (b) drug-free urine, liquid–liquid extraction; (c) drug-free urine, Extrelut clean-up

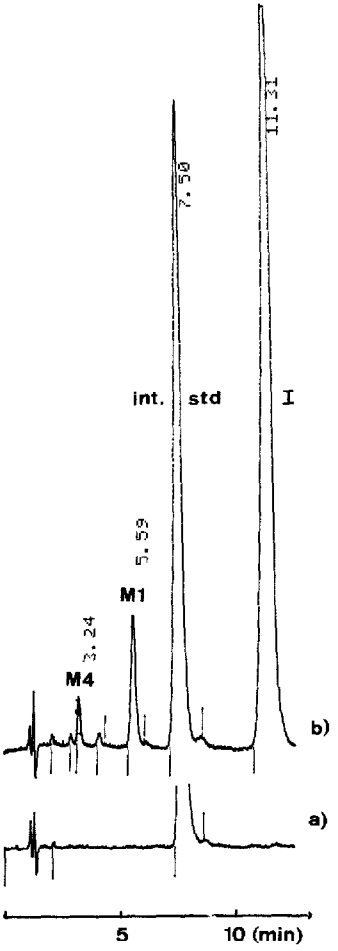

Fig. 4 Chromatograms of plasma samples (a) Predose sample, (b) patient's sample, 12 h after the sixth of repeated intravenous doses of 210 mg of ornidazole to a pediatric patient (body weight, 21 kg, dose interval, 12 h). Concentrations M4 = 110 ng/ml, M1 = 400 ng/ml, ornidazole (I) = 4.35 μg/ml

could be restored, usually, after replacement of the inlet frit. This peak splitting might be due to secondary interactions on the frit material, induced by reagent components or by denatured proteins.

Sample preparation

In the case of small sample volumes (*e.g.* 0.1-ml specimens from neonates), the sample preparation step could be adapted.

Application to biological samples

The method has successfully been applied to the analysis of ornidazole and the

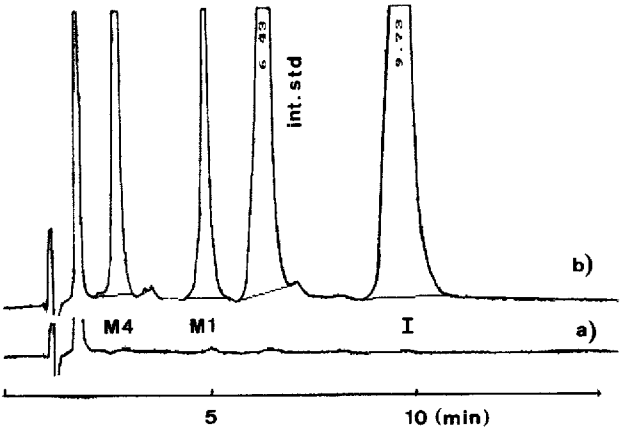

Fig 5 Chromatograms of urine samples: (a) Predose sample; (b) urine fraction 12–24 h after a 1000-mg infusion of ornidazole Concentrations: M4 = 8 8 μg/ml, M1 = 4 1 μg/ml, ornidazole (I) = 21 3 μg/ml

metabolites M1 and M4 in plasma, urine and CSF from clinical samples of adults, children and neonates (Figs. 4 and 5).

CSF concentrations of ornidazole measured up to 24 h following intraveous infusion of 1000 mg of ornidazole to patients with intact meninges exceeded the minimum inhibitory concentration for most anaerobic bacterial strains susceptible to ornidazole.

The method is now under development for human breast milk.

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