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Note

Determination of ornidazole in human plasma and red blood cells using high-performance liquid chromatography

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Ornidazole, a compound of the 5-nitroimidazole class, is used in clinical practice as an antimicrobial agent. In particular, it is active against anaerobic bacteria and is employed in the chemotherapy of various infectious diseases such as *Amoebiasis*, *Lambliasis* and *Thricomoniasis* [1].

Several chromatographic methods for the determination of nitroimidazole derivatives in biological samples are published in the literature. High-performance liquid chromatography (HPLC) has been used for misonidazole [2, 3], metronidazole [3—6], tinidazole [5—7] and nimorazole [8], and gas chromatography (GC) for metronidazole [9,10] and tinidazole [11].

For the determination of ornidazole, an improvement was recently achieved by elimination of the complex clean-up steps previously required for its gas chromatographic detection [12]. The GC method applied to the blood by Bhatia and Shanbhag [13] shows an appreciable accuracy, precision and sensitivity but involves the derivatization of ornidazole and does not consider the metabolites of the drug. The HPLC procedure for plasma described by Merdjan et al. [14] is more rapid and allows the separation of ornidazole from its metabolites M1 and M4 (Fig. 1). The method, however, even if simple, requires extreme care in the preparation of the sample and is reported only for drug determination in 1 ml of plasma.

Here, we describe an HPLC procedure for the determination of ornidazole in 100 μl of plasma and red blood cells. The extraction is performed according to the method applied by Lanbeck and Lindström [5] to metronidazole

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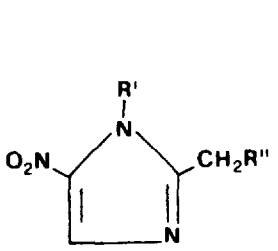

R'	R"	
CH_2CHCH_2Cl (OH)	H	ORNIDAZOLE
CH_2CHCH_2Cl (OH)	OH	METABOLITE M1
H	H	METABOLITE M2
CH_2CHCH_2OH (OH)	H	METABOLITE M4
$CH_2CH_2SO_2CH_2CH_3$	H	TINIDAZOLE (I.S.)

Fig. 1. Structural formulae of 5-nitroimidazole compounds considered.

and tinidazole, with minor modifications. Tinidazole is used as internal standard (I.S.). The method is suitable also for the extraction and separation of ornidazole biotransformation products M1, M2 and M4, which are among the five metabolites identified [15] those strictly related to the nitroimidazole structure.

EXPERIMENTAL

Reagents

Ornidazole and its metabolites M1, M2 and M4 were kindly supplied by Prodotti Roche (Milan, Italy); tinidazole was extracted with diethyl ether from an aqueous alkaline suspension of Fasigin® (Pfizer) tablets; separation and evaporation of the organic solvent provided a crystalline powder, which was checked with the HPLC system described below and used without further purification. Borate buffer (pH 9.0), diethyl ether and methylene chloride (analytical grade) were purchased from Carlo Erba (Milan, Italy), D/4 reagent (a 1.0 *M* aqueous solution of dibutylamine phosphate) from Millipore-Waters (Milan, Italy), acetonitrile (HPLC grade) from Bracco (Milan, Italy). Water was twice-distilled in glass and passed through Porapak Q. The acetonitrile—water—D/4 mixture (mobile phase) was filtered on a 0.5-μm Millipore membrane for organic solvents and deaerated under vacuum in an ultrasonic bath.

Apparatus and chromatographic conditions

A Model 6000 A solvent delivery system and a U6K injector (Waters Assoc.) were used. The column employed (25 cm × 4.6 mm I.D.) was packed with Zorbax C_8 (particle size 5 μm, spherical); the mobile phase (1 ml/min, ca. 100 bar) consisted of acetonitrile—water—D/4 reagent (30:70:1).

The column effluent was monitored at 317 and 229 nm using a high-speed variable-wavelength HP 1040 A detector equipped with a HP 85 B controller, a HP 9121 disc unit and a HP 4740 A plotter (Hewlett-Packard). The signal at 317 nm was recorded with an Omniscribe pen recorder, Model 5119-5A.

Extraction procedure

Plasma or red blood cells from citrated blood (100 μl) were placed in a 10-ml tube and 10 μl of methanol containing 0.1 μg/μl internal standard, 0.5 ml of borate buffer, 1.5 ml of diethyl ether and 1 ml of methylene chloride were added. The mixture was shaken on a Vortex mixer (1 min) and centrifuged

(1000 *g*, 2 min). The upper layer was separated and evaporated to dryness under a stream of nitrogen. The residue was then dissolved in 100 μl of the mobile phase, filtered through a 2-μm Teflon filter tip (Supelco) and injected (5—15 μl) into the chromatograph.

Calibration curves

Two calibration curves were prepared by adding known amounts of ornidazole, equivalent to 1, 5, 10, 15 and 20 μg/ml, to both blank plasma and red blood cells. Five determinations were performed for each point, using the procedure described above. Peak-height ratios (ornidazole versus tinidazole) were plotted against ornidazole concentrations.

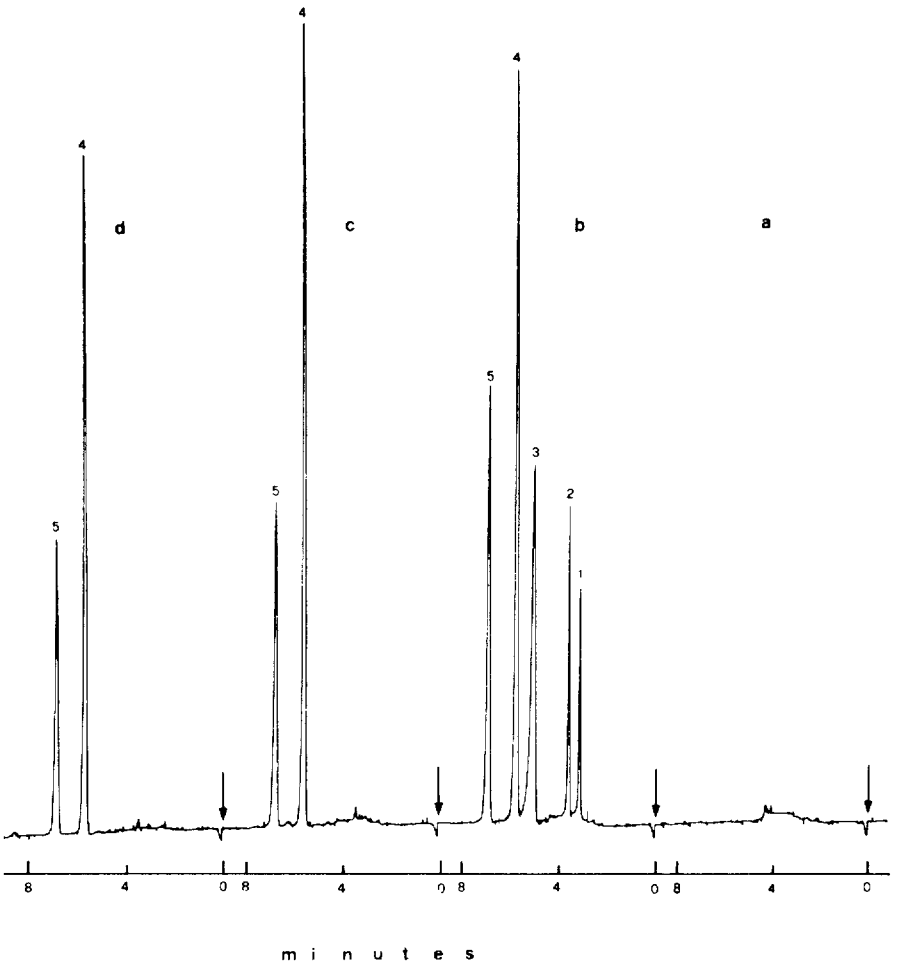

Fig. 2. Chromatograms obtained from: (a) blank human plasma; (b) blank human plasma spiked with ornidazole (5 μg/ml), ornidazole metabolites M1 (10 μg/ml), M2 (2.5 μg/ml), M4 (10 μg/ml) and I.S. (10 μg/ml); (c) and (d) plasma and red blood cells 12 h after an intravenous injection of 500 mg of ornidazole. Injection volume: 15 μl. Peaks: 1 = metabolite M4; 2 = metabolite M2; 3 = metabolite M1; 4 = I.S.; 5 = ornidazole.

RESULTS AND DISCUSSION

The chromatograms obtained when ornidazole and its metabolites M1, M2 and M4 were extracted from plasma and red blood cells are shown in Fig. 2. The retention times of the peaks were: 3.01 (M4), 3.46 (M2), 4.91 (M1), 5.68 (I.S.) and 6.88 (ornidazole) min.

Fig. 3a and b includes elaborations of a chromatogram obtained from the red blood cells prepared as in Fig. 2. To check the purity of the peaks of ornidazole and the I.S., UV spectra of the compounds at three different times corresponding to "up", "apex" and "down" of the interested peaks were recorded during HPLC analysis and superimposed (Fig. 3a); superimposition was also made for the signals at 229 and 317 nm (Fig. 3b).

Table I shows the precision and accuracy obtained for ornidazole determinations. The equations that describe the standard curves, determined by least-squares regression analysis, were $y = 0.1211\ x + 0.0027$ and $y = 0.1206\ x + 0.0070$ for plasma and red blood cells, respectively. The corresponding correlation coefficients (r) were 0.9992 and 0.9988.

The limit of quantitation of ornidazole was 0.3 μg/ml with 0.1 ml of biological sample and the detector set at 317 nm; to reach this limit, at least 40 μl of the extract must be injected and a suitable concentration of I.S. used (e.g. 1 μg/ml). The tinidazole concentration (10 μg/ml) employed for the calibration curves corresponds to that routinely used in pharmacokinetic studies, since the ornidazole plasma levels exceed 1 μg/ml for at least 36 h after a single intravenous [14] or oral [16] administration.

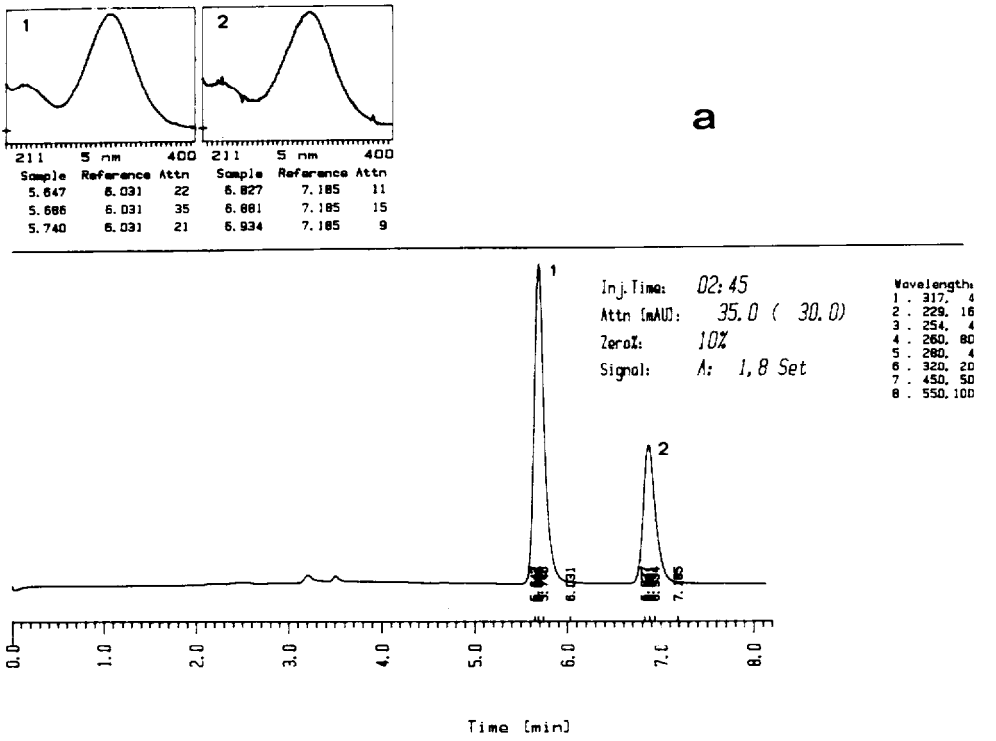

Fig. 3.

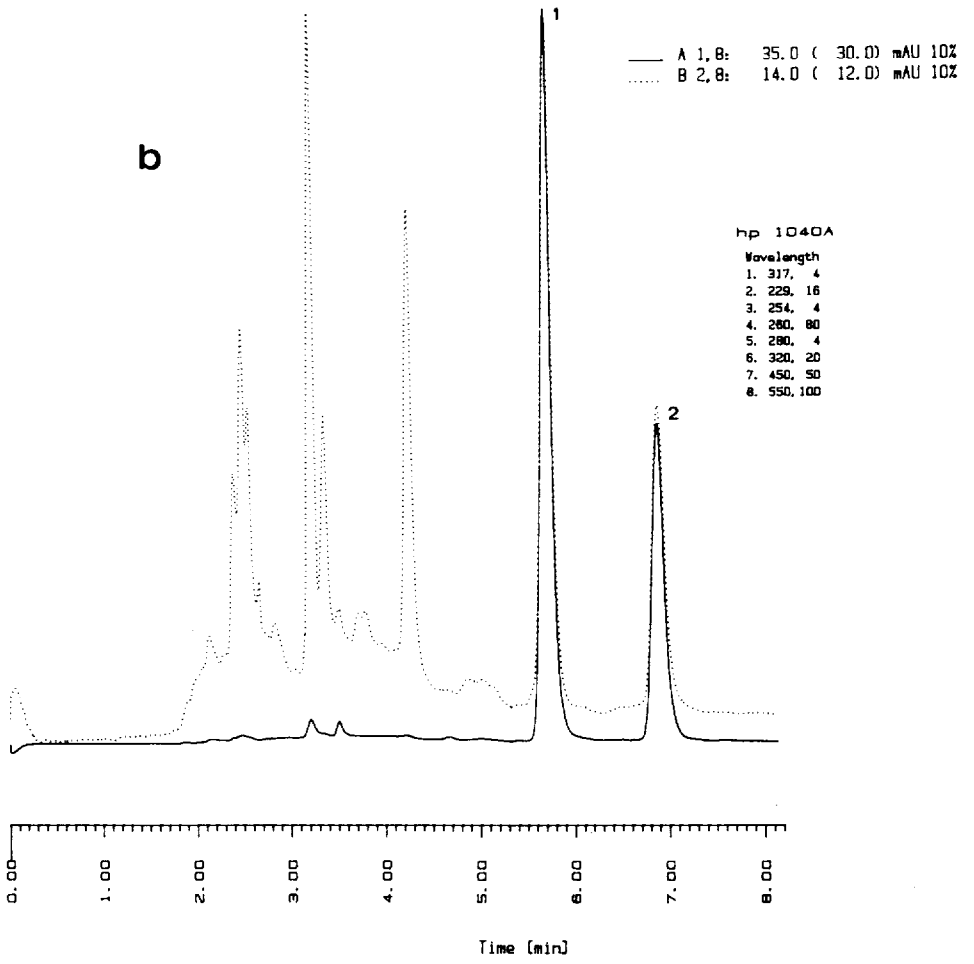

Fig. 3. (a) Chromatogram and UV spectra from red blood cells; (b) superimposition of the signals recorded at 317 (A) and 229 nm (B). Plotter: HP 7470 A; injection volume: 25 µl. Peaks: 1 = I.S., 2 = ornidazole.

TABLE I

ACCURACY AND PRECISION OF METHOD

Plasma			Red blood cells		
Amount added (µg/ml)	Amount found (µg/ml, mean ± S.D.)	Coefficient of variation (%)	Amount added (µg/ml)	Amount found (µg/ml, mean ± S.D.)	Coefficient of variation (%)
1	0.97 ± 0.02	2.4	1	0.94 ± 0.03	2.9
5	4.98 ± 0.04	0.8	5	5.01 ± 0.27	5.3
10	10.12 ± 0.20	2	10	10.01 ± 0.15	1.5
15	14.93 ± 0.22	1.5	15	14.97 ± 0.13	0.9
20	19.99 ± 0.57	2.9	20	19.90 ± 0.74	3.7

Contrary to the observations of other authors [14], who reported a doubling of the drug peak at a pH value above 5.5, alkalinization of the sample (pH 9.0) during the extraction procedure does not cause decomposition of ornidazole. Furthermore, the check of peak purity was satisfactory both for ornidazole and tinidazole.

Interferences by endogenous compounds or other nitroimidazole derivatives (nimorazole, metronidazole, misonidazole) were never observed. The metabolites are perfectly resolved from the parent compound and the I.S.

Ornidazole metabolites were never revealed in human blood samples collected at any time after a single intravenous dose of 500 mg of ornidazole. Only the parent compound was detected at concentrations of 11.28, 7.87, 7.27, 5.87 and 3.59 μg/ml in plasma and 9.28, 8.91, 8.61, 6.46 and 3.86 μg/ml in red blood cells, 1, 2, 3, 6 and 12 h after drug administration, respectively.

In conclusion, the HPLC procedure reported here for the determination of ornidazole in human plasma and red blood cells is rapid, reproducible, specific and sensitive and can be applied to drug measurements in pharmacokinetic studies without interference from its metabolites M1, M2 and M4.

Preliminary investigations indicate that the procedure is extensible to other biological specimens such as biopsy samples and purulent abscesses.

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