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Short communication

Simple and sensitive method for determination of metronidazole in human serum by high-performance liquid chromatography

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Abstract

A simple and sensitive HPLC method for determination of metronidazole in human plasma has been developed. A step of freezing the protein precipitate allowed an efficient separation of aqueous and organic phases minimizing the noise level and improved therefore the limit of quantitation (10 ng ml^{-1} using 1 ml of plasma sample). The separation of compounds was performed on a RP 18 column with acetonitrile–aqueous 0.01 *M* phosphate solution (15:85, v/v) as mobile phase. Detection was performed by UV absorbance at 318 nm. Metronidazole was well resolved from the plasma constituents and internal standard. An excellent linearity was observed between peak-height ratios plasma concentrations over a concentration range of 0.01 to 10 $\mu g\ ml^{-1}$. Within-day and between-day precision (expressed by relative standard deviation) and accuracy (mean error in per cent) did not exceed 4% between 1 and 10 $\mu g\ ml^{-1}$ and 8.3 and 7.2% respectively for the limit of quantitation. The method is suitable for bioavailability and pharmacokinetic studies in humans. © 1998 Elsevier Science B.V. All rights reserved.

Keywords: Human plasma; Metronidazole

1. Introduction

Metronidazole [1-(hydroxyethyl)-2-methyl-5-nitroimidazole] is a drug used for the treatment of protozoal infections and infections caused by anaerobic micro-organisms. A large number of high-performance liquid chromatographic methods have been described to analyze metronidazole in body fluids [1–11]. Nevertheless, the limit of quantitation was not given [1–5,7,8,11] or given without validation [6,9] or with a high value at 500 ng ml^{-1} (using 0.1 ml of plasma sample) [10]. The method developed in this paper allowed to validate a limit of the quantitation at 10 ng ml^{-1} using 1 ml of plasma sample.

2. Materials and methods

2.1. Chemicals

Metronidazole and tinidazole were purchased from

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Sigma (Saint Quentin Fallavier, France). Potassium dihydrogen phosphate was obtained from Merck (Darmstadt, Germany). HPLC grade acetonitrile was purchased from SDS (Peypin, France).

2.2. Chromatographic system

The chromatography was carried out using an HP 1050 instrument (Hewlett–Packard, Les Ulis, France) equipped with a power supplier, an autosampler and a UV spectrophotometric detector connected to a data collection system. The analytical column used was a LiChrospher 100 RP 18 (5 μm particle size, 125×4 mm) from Merck. The mobile phase, consisted of acetonitrile–0.01 *M* phosphate solution (KH_2PO_4), pH 4.7, 15:85, v/v, was degassed and filtered through a 0.45 μm filter (Millipore, Saint-Quentin, Yvelines, France). The flow-rate was 1 ml min^{-1}. The detection wavelength was 318 nm. The injection volume was 20 μl and the run time was 6 min.

2.3. Calibration standards

A stock solution of metronidazole was prepared by dissolving 100 mg in 100 ml of deionized water. This solution was used to prepare working standard solutions daily for different concentration between 0.1 mg ml^{-1} and 0.1 μg ml^{-1} by dilution in deionized water.

Calibration samples of metronidazole were prepared in blank plasma. The plasma (1 ml) was supplemented with working standard solutions of metronidazole at 0.01, 0.025, 0.05, 0.1, 0.5, 1, 2.5, 5 and 10 μg ml^{-1}.

2.4. Extraction procedure

In a 5 ml glass tube, a plasma sample (1 ml) was mixed with 50 μl of the aqueous tinidazole internal standard (100 μg ml^{-1}). Acetonitrile (1.5 ml) was added and the sample was vortex-mixed for 5 min and then centrifuged at 1600 *g* for 5 min. The tubes were capped and kept frozen at −20°C for 20 min.

An aliquot (1 ml) of the surpernatant was transferred to a 5 ml glass tube and the solvent was evaporated to dryness using a Speed–Vac concentrator. The sample was reconstituted in 0.5 ml of mobile phase before injection onto the HPLC system.

2.5. Biological application

To demonstrate the applicability of the procedure for measurement of metronidazole levels in pharmacokinetic studies, 500 mg of metronidazole was orally administered to four healthy male volunteers. Blood samples were collected at 0, 0.25, 0.50, 0.75, 1, 1.50, 2, 3, 4, 5, 7, 9, 12, 16, 24 and 36 h following oral administration. Blood samples were centrifuged and plasma were kept frozen at −20°C until analysis.

3. Results and discussion

Fig. 1 shows typical chromatograms of a blank plasma, a plasma spiked with metronidazole (5 μg ml^{-1}), a plasma spiked with tinidazole (IS at 5 μg ml^{-1}) and a sample obtained from a volunteer after oral administration of 500 mg of metronidazole at 1 h. Metronidazole and IS were well resolved and the retention times were 2.6 and 4.8 min, respectively. No interfering peaks were observed in the chromatogram of the blank human plasma.

The peak–area ratios of metronidazole to the internal standard were calculated and used to construct calibration lines of peak–area ratio (y) against the respective sample concentration (x). Linear regression analysis was used to calculate the slope, intercept and correlation coefficient (r) of calibration lines. The linearity was found to be quite satisfactory and reproducible with time. The equations of mean linear regression ($n=6$) were $y=0.215x+0.00101$ ($r=0.9998$) for total range (0.01 to 10 μg ml^{-1}) and $y=0.204x+0.012$ ($r=0.9990$) for low range (0.01 to 0.1 μg ml^{-1}).

The limit of quantitation (LOQ) was determined after analyzing ten blank plasma samples. The mean calculated value ±SD was 1.64±0.45 ng ml^{-1}. The limit of detection, generally defined [12] as the mean of the ten blank signals plus three times the SD of the blank, was 3 ng ml^{-1} and the theoretical limit of quantitation as the mean plus ten times the SD of the blank, was 6 ng ml^{-1}. The mean of ten blank plasma

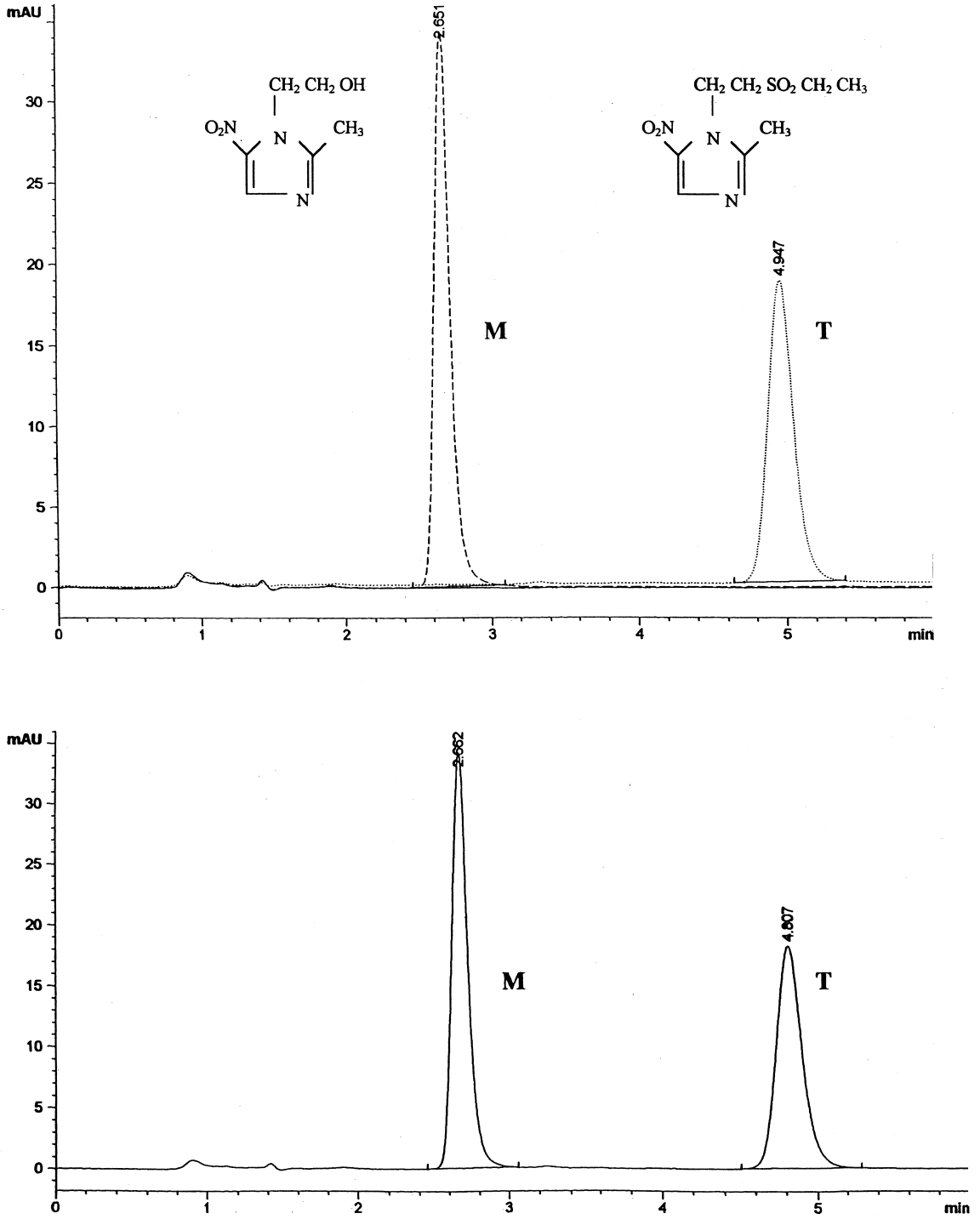

Fig. 1. (a) Chromatograms of a blank plasma [—], a plasma spiked with 5 μg ml^{-1} of metronidazole (M) [----] and a plasma spiked with 5 μg ml^{-1} of tinidazole (T) [....]. (b) Chromatogram of a sample obtained from a volunteer after oral administration of metronidazole (500 mg) at 1 h.

Table 1
Precision and accuracy of metronidazole assay in plasma

Theoretical concentration ($\mu g\ ml^{-1}$)	Calculated concentration (mean±SD) ($\mu g\ ml^{-1}$)	Precision[a] (%)	Accuracy[b] (%)
Intra-assay ($n=10$)			
0.01[c]	0.0107±0.0009	8.2	+7.2
1	0.97±0.009	1.0	−2.9
10	10.13±0.112	1.1	+1.3
Inter-assay ($n=6$)			
0.01[c]	0.0105±0.0009	8.3	+4.6
1	0.96±0.023	2.3	−3.6
10	10.00±0.076	0.8	+0.01

[a] Relative standard deviation=[standard deviation (SD)/mean]×100.
[b] Relative error=[(found−added)/added]×100.
[c] Limit of quantitation.

supplemented at 10 $ng\ ml^{-1}$ was 10.73±0.89 $ng\ ml^{-1}$ with a precision (RSD) of 8.2% and an accuracy (mean error in per cent) of 7.2%. Inter-assay precision and accuracy determined on six separate days at 10 $ng\ ml^{-1}$ were 8.3 and 4.6% respectively. A concentration of 10 $ng\ ml^{-1}$ was therefore considered as a validated limit of quantitation.

Precision and accuracy were likewise determined at 1 and 10 $\mu g\ ml^{-1}$. Quite satisfactory results were obtained for both intra and inter-assays (Table 1) with a precision (RSD) <3% and an accuracy (mean error in per cent) <4%.

The results of the stability study, expressed as mean concentration (±SD) and determined at various time intervals of storage at −20°C, are reported in Table 2. The stability of plasma samples kept frozen for three months appears to be satisfactory (mean relative errors were 6.3, 0.3 and 4.1% respectively for 0.05, 1 and 10 $\mu g\ ml^{-1}$, $n=2$).

Plasma concentration–time courses of metronidazole in four healthy subjects after single oral dose of 500 mg are shown in Fig. 2. The metronidazole was rapidly absorbed. A maximum plasma concentration at 7.7 $\mu g\ ml^{-1}$ was reached between 1 h and 3 h. The half-life of metronidazole was about 8.80 h .These findings are in agreement with previous studies [13].

In summary, a rapid, sensitive HPLC method was described for the determination of metronidazole in human plasma. Protein precipitation with acetonitrile followed by a step of freezing which allowed an efficient separation of aqueous and organic phases and therefore improved the limit of quantitation. Polar interferences are therefore decreased which affords a gain in signal to noise ratio and column life

Table 2
Stability of metronidazole in plasma

	Means of calculated concentrations ($n=2$) with SD and (mean relative errors)		
Days of storage	For theoretical levels ($\mu g\ ml^{-1}$)		
at −20°C	0.050	1.00	10.00
J 0	0.053±0.008 (+6.0)	0.95±0.02 (−5.0)	9.77±0.03 (−2.3)
J 2	0.049±0.002 (−2.0)	0.98±0.01 (−2.0)	10.34±0.39 (+3.4)
J 8	0.059±0.003 (+18.0)	1.07±0.01 (+7.0)	10.94±0.14 (+9.4)
J 15	0.058±0.004 (+16.0)	0.99±0.02 (−1.0)	10.23±0.26 (+2.3)
J 22	0.058±0.004 (+16.0)	0.97±0.01 (−3.0)	10.11±0.27 (+1.1)
J 30	0.056±0.006 (+12.0)	1.06±0.00 (+6.0)	10.80±0.25 (+8.0)
J 93	0.041±0.002 (−18.0)	0.96±0.03 (−4.0)	10.66±1.28 (+6.6)

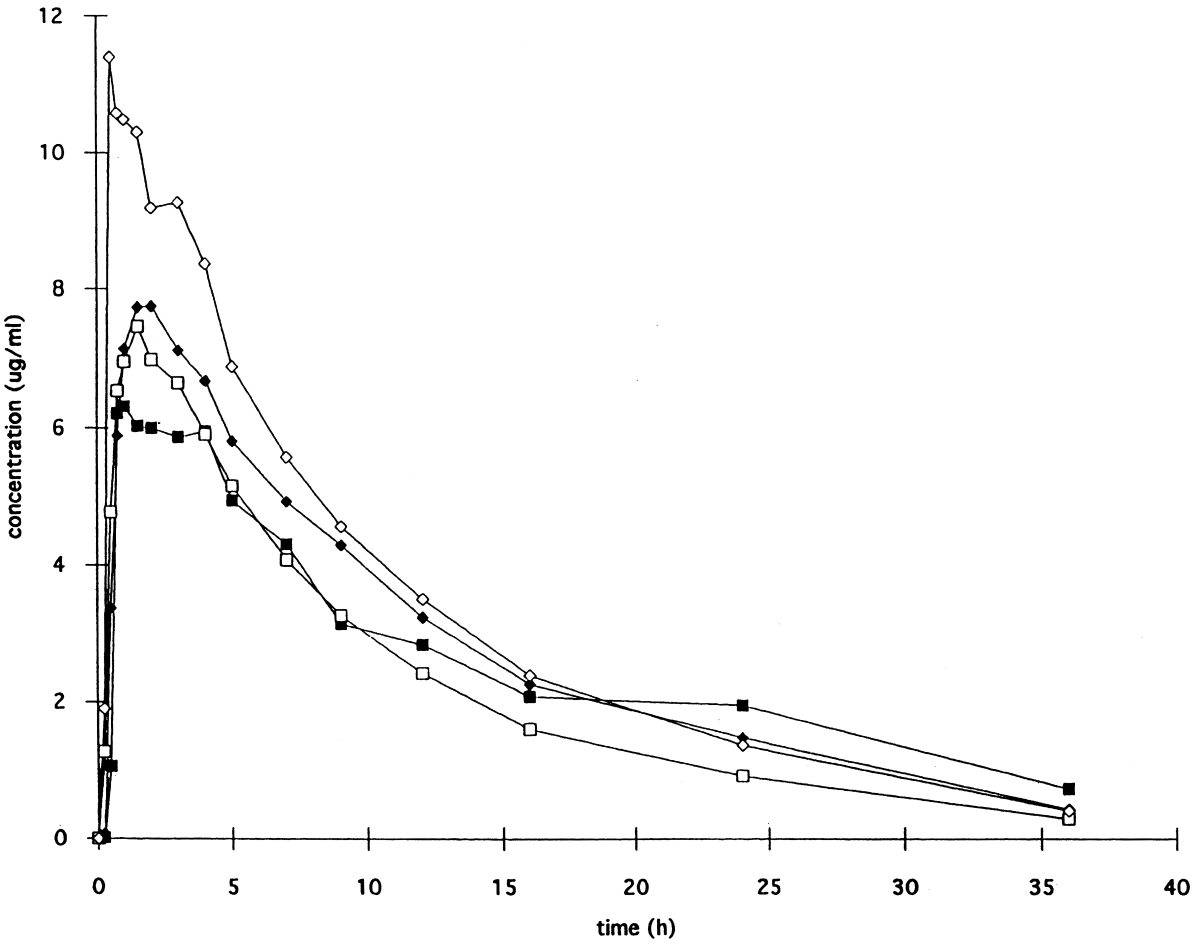

Fig. 2. Plasma concentration–time profiles of metronidazole in four healthy subjects after single oral dose of 500 mg.

is likewise greatly extended. This analytical procedure is readily applicable to routine analysis of plasma samples for bioavailability and pharmacokinetic studies in human. In clinical situations, other substances may be co-administered, and possible interfering drugs would have to be investigated. However, detection is carried out at 318 nm and a lot of drugs are known not to absorb at this wavelength [10], minimising possible interference.

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