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Note

Determination of metronidazole and tinidazole in plasma and feces by high-performance liquid chromatography

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Metronidazole (Fig. 1a) is an antimicrobial agent used for treatment of some

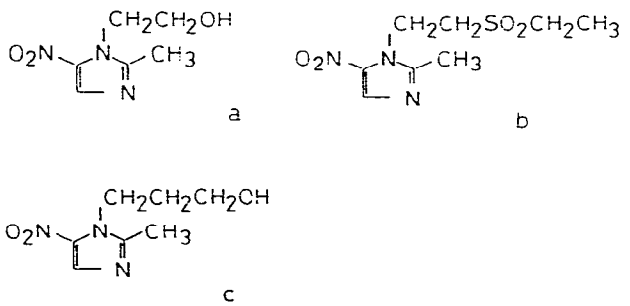

Fig. 1. Structural formulae: a = metronidazole; b = tinidazole; c = 1-(3-hydroxypropyl)-2-methyl-5-nitroimidazole, internal standard.

infectious diseases, mainly infections caused by *Trichomonas vaginalis*, *Endamoeba histolytica* and *Giardia lamblia*. The drug tinidazole (Fig. 1b) has similar indications. These two drugs have been assayed by biological polarographic [1, 2] and colorimetric [3,4] methods. For metronidazole, gas chromatographic methods have been described [5,6]. The liquid chromatographic (LC) method described here was developed in connection with a clinical trial on Crohn's disease with metronidazole. The reason was that the biological assay tended to give lower values after storage of frozen samples and was not sensitive enough for many of the samples. The polarographic method was too unspecific. The

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gas chromatographic methods were considered too tedious, because relatively large solvent volumes had to be handled, and too insensitive due to background interference.

The LC method is based on a reversed-phase system and UV detection. Both metronidazole and tinidazole are analysed on the same system and either may be used as internal standard for the other. In the present method 1-(3-hydroxypropyl)-2-methyl-5-nitroimidazole (Fig. 1c) was also used as internal standard for metronidazole.

During the preparation of this paper an assay for tinidazole in plasma was published using high-performance liquid chromatography (HPLC) [7]. This assay uses no internal standard and the sensitivity is about twenty times less than in the method described here. One reason for the difference in sensitivity may be that the chromatographic system is not very suitable for tinidazole which is indicated by the broad tailing peak in the chromatogram published.

EXPERIMENTAL

System

The liquid chromatograph used consisted of a pump Model M 600, a U6K injector from Waters Assoc. (Milford, Mass., U.S.A.) and a UV detector Model 770 from Spectra-Physics. The column (0.15 m × 4.6 mm I.D., stainless steel) was slurry-packed with Spherisorb S5 ODS (particle size 5 μm). The mobile phase consisted of 0.01 *M* phosphate buffer (pH 5.5) mixed with 15% acetonitrile operated at a flow-rate of 1 ml/min at room temperature (ca. 20—22°). The detector wavelength setting was 320 nm.

Extraction

Plasma. To 100 μl of plasma in a 10-ml screw-capped tube were added 0.5 ml 0.1 *M* carbonate buffer, pH 9, and 100 μl internal standard solution. The internal standard solution contained 10 μg/ml of 1-(3-hydroxypropyl)-2-methyl-5-nitroimidazole or 15 μg/ml of tinidazole when metronidazole was analysed and 10 μg/ml of metronidazole when tinidazole was analysed. The extraction took place on a shake board (15 min) with ether—methylene chloride (1.5:1, 2 × 3 ml) followed by centrifugation (500 *g*, 10 min). The extracts were combined and evaporated in a stream of nitrogen. The residue was dissolved in 250 μl of the eluent mixture. After filtration through a pasteur pipette containing fine glass wool, 20 μl was injected into the liquid chromatograph.

Feces. To 20 mg of freeze-dried feces was added 0.5 ml 0.1 *M* carbonate buffer, pH 9, and the mixture was exposed to ultrasonic treatment for 5 min. Extraction was carried out as above except that an exact volume of the organic phase was evaporated. The residue obtained after evaporation of the solvents was dissolved in 250 μl of the eluent mixture. The solution was filtered through a pasteur pipette packed in the following order: fine glass wool, Celite 10 mg (Johns-Manville, Lompoc, Calif., U.S.A.) and glass wool in the narrow part of the pipette. 40 μl were injected into the liquid chromatograph.

Preparation of 1-(3-hydroxypropyl)-2-methyl-5-nitroimidazole internal standard (IS)

1,3-Propane-diol was monobenzylated and reacted with *p*-toluene sulfonyl-chloride [8]. The resulting benzyloxypropyl *p*-toluene sulfonate was reacted with 2-methyl-5-nitroimidazole [9]. After removal of the benzyl group, the hydrochloride of the IS was precipitated in ethanol with ether—HCl and filtered off.

RESULTS

The recovery of 5 μg/ml of metronidazole, tinidazole or the IS after extraction from plasma was slightly above 90% for metronidazole and almost quantitative for tinidazole and IS.

The reversed-phase system used gave a low background chromatogram with no interfering peaks. Fig. 2a shows the chromatogram obtained from the analysis of a plasma sample containing 5 μg/ml of metronidazole and IS. Fig. 2b shows the chromatogram obtained from the analysis of a plasma sample containing 5 μg/ml of tinidazole. Fig. 2c shows the analysis of a blank plasma sample. IS was preferred as internal standard in the analysis of metronidazole because of its structural similarity and close retention time which made the analysis relatively fast.

Since the output of the UV detector is linear in absorbance units, the area under the peak is proportional to the concentration. Two calibration graphs were constructed for metronidazole by analysing samples to which 0—16 μg/ml of metronidazole had been added and plotting the peak area ratios (metronidazole:internal standard) versus the concentration. For one graph IS was used as internal standard and for the other tinidazole. Both graphs were linear and passed through the origin. The standard deviation was determined by analysing 8 samples to which 7.5 μg/ml of metronidazole had been added and was found to be about 1.5% with either of the internal standards.

Plasma samples were prepared in order to evaluate the lower limit for quantification of metronidazole and it was found to be about 25 ng/ml provided 50%

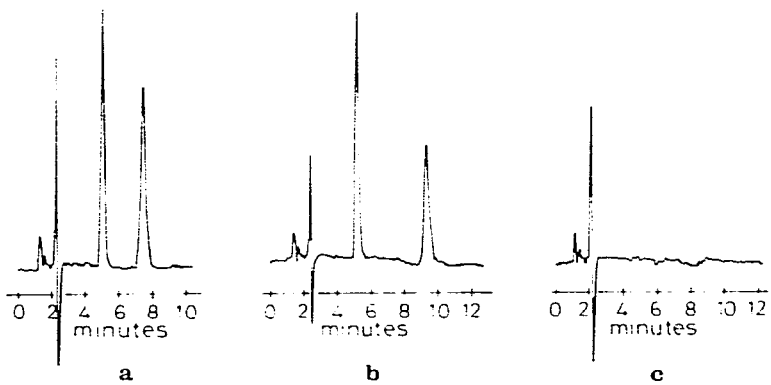

Fig. 2. Chromatograms showing; (a) plasma sample containing metronidazole and IS, (b) plasma sample containing metronidazole and tinidazole, (c) blank plasma sample.

of the extracted amount was injected into the liquid chromatograph.

A calibration graph for the plasma determination of tinidazole was prepared by the same procedure as that for metronidazole. The standard deviation was 3% (5 samples, concn. = 7.5 μg/ml). The response of the detector to tinidazole was about half that of metronidazole.

The determination in feces was only carried out with metronidazole and no internal standard was used. The chromatograms in Fig. 3a and b result from the analysis of a feces sample containing 49 ng/mg metronidazole and a blank feces sample respectively (aliquot of sample injected in Fig 3b, 2.7 times larger than that in 3a). The chromatograms of feces samples were surprisingly free of background peaks. Extra peaks sometimes appeared but in about 50 different analyses none have interfered with the metronidazole peak. The calibration graph for feces determinations was constructed by adding 0—500 ng in 500 μl water to 20-mg samples of freeze-dried feces and exposing them to ultrasonic treatment for 10 min. They were then allowed to stand for 20 min before the analysis described above. The peak areas obtained were plotted against the concentration. The standard deviation was determined by analysing 6 samples containing 10 ng/mg of dry feces and was found to be 2.6%.

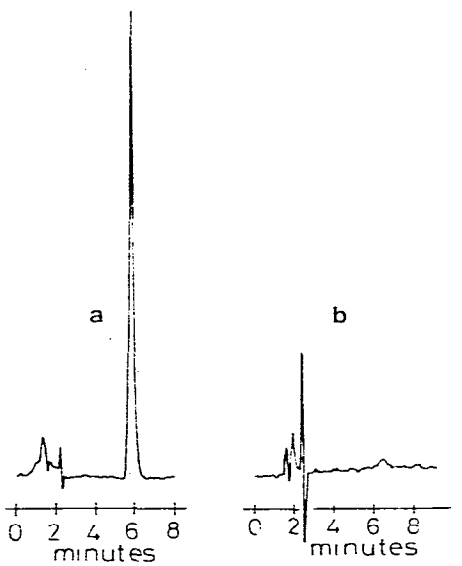

Fig. 3. Chromatograms showing; (a) feces sample containing metronidazole, (b) blank feces sample.

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