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DETERMINATION OF METRONIDAZOLE, MISONIDAZOLE AND ITS METABOLITE IN SERUM AND URINE ON RP-18 HIGH-PERFORMANCE THIN-LAYER CHROMATOGRAPHIC PLATES

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SUMMARY

A method for the determination of radiotherapeutic concentrations of metronidazole, misonidazole and its metabolite is described. The biological fluid (serum or urine) was deproteinized with acetone containing 2-nitroimidazole as internal standard, centrifuged and the supernatant evaporated under vacuum. The residue, dissolved in acetone, was applied to an HPTLC-RP-18 layer and, after development, quantitation was achieved with a scanning densitometer. The response was linear up to 180 μg/ml for all tested compounds and the detection limit was 0.5 μg/ml. Owing to its rapidity and sensitivity the method can be considered to be equivalent to high-performance liquid chromatography.

INTRODUCTION

Metronidazole and misonidazole are widely employed as radiosensitizing drugs in the radiotherapy of hypoxic tumour cells. As both the radiosensitizing effect and the toxicity of these drugs are dose-dependent, their determination in biological fluids is important. Many analytical techniques are available for the quantitation of these compounds, such as UV spectrometry [1], polarography [2], gas—liquid chromatography [3], thin-layer chromatography (TLC) [4] and high-performance liquid chromatography (HPLC) [5, 6]. Spectropho-

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tometric and polarographic techniques are rapid and sensitive but the resolution of misonidazole and its metabolite is, in practice, not satisfacory [3]. This separation can be achieved by chromatographic methods, of which HPLC is considered, at present, to be the most suitable for routine analyses [7].

This paper describes the application of bonded-phase TLC to the determination of metronidazole, misonidazole and its O-demethylated metabolite in biological fluids and demonstrates that this method is as suitable as HPLC for routine analyses.

EXPERIMENTAL

Chemicals

All chemicals and solvents were of analytical-reagent grade. Metronidazole was obtained from Farmitalia (Milan, Italy) and misonidazole and desmethylmisonidazole from Hoffmann-La Roche (Basle, Switzerland). 2-Nitroimidazole was supplied by Aldrich Europe (Beerse, Belgium).

Apparatus

All measurements were carried out with a Camag (Muttenz, Switzerland) TLC/HPTLC 76500 scanner reading the absorbance of the spots at 320 nm. In all determinations the instrument was zeroed on a blank area of the layer and a uniform baseline was always observed. The peaks obtained were quantitatively integrated by a Spectra Physics (Darmstadt, G.F.R.) Minigrator.

Sample preparation

The following procedure was found to be suitable for samples of urine and blood serum. The sample (1 ml) was mixed with acetone (2 ml) containing 2-nitroimidazole as an internal standard (22.6 μg/ml) and shaken for 1 min. After centrifugation for 10 min at 1200 *g* (Hettich refrigerated centrifuge) the supernatant was removed and dried in a rotary vacuum evaporator. The residue was dissolved in 0.5 ml of acetone.

Chromatography

A 1-μl sample was spotted quantitatively on an HPTLC-RP-18 bonded-phase layer (Merck, Darmstadt, G.F.R.) with a Camag micro-applicator. The layers were developed at room temperature using the ascending or horizontal technique according to Kristensen [8]. The best developing solvent system was *n*-hexane—acetone—96% ethanol (19:6:1). The layer was air dried and the spots were quantitated by scanning densitometry.

RESULTS AND DISCUSSION

HPTLC-RP-18 layers were chosen for the separation of the nitroimidazole radiosensitizers because the resolution obtained on conventional silica gel plates was not satisfactory with any of the solvent systems used. The optimal mobile phase was selected by performing preliminary chromatographic runs with some of the commonly used solvent mixtures for reversed-phase layers, viz., methanol—water, acetonitrile—water and isopropanol—water. With these

developing solvents no separation was achieved and all spots were close to the solvent front. A slight separation and a slight decrease in the R_F values occurred when the eluent mixture was isopropanol—*n*-hexane (1:1) (R_F values: misonidazole 0.85, desmethylmisonidazole 0.85, metronidazole 0.70 and 2-nitroimidazole 0.93).

Taking these results into consideration, it was necessary to decrease further the elution strength of the mobile phase to obtain good separations. The solvent system that gave the most satisfactory results for the separation of the nitroimidazole compounds and compactness of the spots was *n*-hexane—acetone—96% ethanol (19:6:1).

Fig. 1A shows a chromatogram of a mixture of desmethylmisonidazole, metronidazole, misonidazole and the internal standard 2-nitroimidazole obtained on the HPTLC-RP-18 layers with the selected eluent. Under these conditions all R_F values are reproducible, as can be seen in Table I. The four peaks were completely resolved with this solvent system, whereas Marques et al. [9] did not succeed in separating metronidazole from misonidazole with their HPLC method. The nitroimidazoles can be also separated using the solvent system *n*-hexane—acetone (3:1), although metronidazole and desmethylmisonidazole are not completely resolved. However, a better separation can be achieved by double development. Comparison of Fig. 1A and B shows that the blank serum, taken from a patient with a bladder tumour

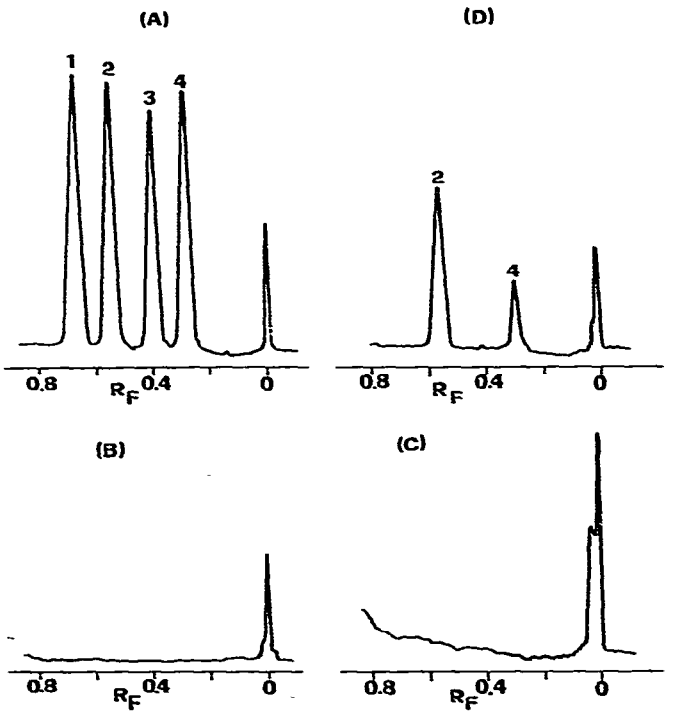

Fig. 1. Chromatograms of (A) a mixture of 0.28 μg of internal standard (peak 1), 0.50 μg of misonidazole (peak 2), 0.43 μg of metronidazole (peak 3) and 0.47 μg of desmethylmisonidazole; (B) serum blank; (C) urine blank; and (D) serum sample of a patient taken 5 h after oral administration of 0.6 g/m² of misonidazole.

TABLE I

R_F × 100 VALUES FOR 8-cm RUNS AND RESOLUTION FACTORS

Each value is the mean (± S.D.) of five determinations.

Compound	R_F × 100	Resoltuion factor*
Desmethylmisonidazole	31 ± 1	
Metronidazole	41 ± 1	1.4
Misonidazole	58 ± 1	2.4
2-Nitroimidazole	68 ± 1	1.5

*Defined as $\frac{2\Delta x}{w_2+w_1}$ where Δx is the distance between two peaks and w_2 and w_1 are peak widths.

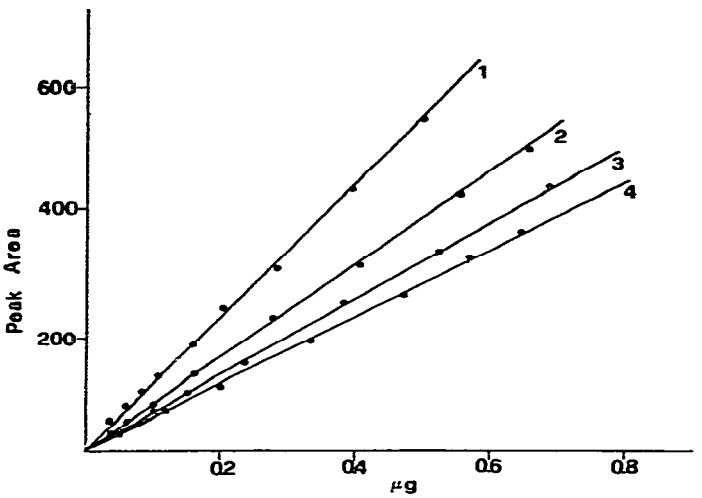

Fig. 2. Calibration graphs for (1) internal standard, (2) metronidazole, (3) desmethylmisonidazole and (4) misonidazole. Each point is the mean of five determinations.

immediately before administration of misonidazole, contains no components capable of interfering with peaks 1—4. A similar result was obtained for extracts of control urine (Fig. 1C). In contrast, when extracts of urine samples are chromatographed on silica gel some UV-absorbing constituents move with tailing, thus interfering in the quantitation of desmethylmisonidazole. Fig. 1D shows a chromatogram of an acetone extract of serum taken from the same patient 5 h after oral administration of misonidazole (0.6 g/m^2), and misonidazole (peak 2) and its O-demethylated metabolite (peak 4) are present.

Calibration graphs for the determination of 1—4 by TLC were prepared by spotting different amounts of a mixture of the nitroimidazoles and plotting peak areas against concentration. As shown in Fig. 2, all calibration graphs were linear up to 3.7 nmole (corresponding to about 180 μg/ml in the sample). The precision was determined for misonidazole, desmethylmisonidazole and metronidazole at 65, 20 and 65 μg/ml, respectively, and gave $\bar{x}$ = 64.7 μg/ml (C.V. = 2%, n = 5), $\bar{x}$ = 19.8 μg/ml (C.V. = 3.3%, n = 5) and $\bar{x}$ = 65.2 μg/ml

TABLE II

RECOVERIES ON EXTRACTION OF MISONIDAZOLE, METRONIDAZOLE AND DESMETHYLMISONIDAZOLE FROM SERUM AND URINE

Each value is the mean of five determinations.

Compound	Added (μg/ml)	Recovery from serum (%)	Recovery from urine (%)
Misonidazole	30.0	97.3	98.2
	70.0	98.4	98.9
	100.0	94.8	97.1
	130.0	96.5	95.8
Mean ± S.D.		97.1 ± 1.4	
Metronidazole	30.0	93.2	94.5
	50.0	94.2	94.8
	80.0	92.9	94.3
	110.0	93.6	93.5
Mean ± S.D.		93.9 ± 0.8	
Desmethylmisonidazole	5.0	94.1	95.8
	10.0	95.9	93.9
	20.0	95.2	94.4
	30.0	94.7	93.5
Mean ± S.D.		94.7 ± 1.2	

(C.V. = 1.7%, n = 5), respectively. Allowing a minimum signal-to-noise ratio of 2, the detection limit was ca. 0.5 μg/ml (in serum or urine samples) for each compound. Recovery experiments were carried out by adding known amounts of each nitroimidazole to serum and urine samples and processing the samples as described under Experimental. As shown in Table II, the overall recoveries were good. The concentration of misonidazole in serum after oral administration of 0.6 g/m^2 of misonidazole to a patient with a bladder tumour determined by the present method was compared with that obtained by polarography. Good agreement was observed, indicating that the proposed method can be utilized in routine analyses to determine the levels of nitroimidazole radiosensitizers in biological fluids.

A comparison of the proposed technique with the HPLC and polarographic techniques currently employed to determine the levels of misonidazole and desmethylmisonidazole in tissues and body fluids is shown in Table III. It can be seen that the TLC and HPLC methods are equivalent as far as sensitivity and specificity are concerned. Also, the analysis times are similar for these two methods, as eight samples can be processed in about 2 h when the TLC method is employed. After a measurement the layer can be developed with 96% ethanol to obtain complete elution of the sample compounds and then re-utilized for a new measurement. The washed layer gives reproducible results and this procedure can be utilized until the baseline remains sufficiently uniform. Another disadvantage with silica gel plates is that washing with ethanol is not adequate to remove completely interfering substances in the urine extracts, thus precluding further runs on the same plate.

TABLE III

COMPARISON OF RESULTS OBTAINED IN ASSAYING MISONIDAZOLE AND DESMETHYLMISONIDAZOLE IN SERUM SAMPLES BY TLC, HPLC AND DIFFERENTIAL-PULSE POLAROGRAPHY

Compound	TLC			HPLC*	
	R_F	Resolution	Detection limit (μg/ml)	Retention time (min)	Resolution
Misonidazole	0.58	good	≈0.5	4.1	good
Desmethylmisonidazole	0.31		≈0.5	2.3	

*Data taken from ref. 5.
**Experimental conditions: 7 ml of ethanol were added to 1 ml of serum; after centrifugation, the supernatant was diluted with an equal volume of 0.1 *M* potassium chloride solution.
***Same conditions as above, but with addition of Tylose up to a concentration of 0.1% (w/v).

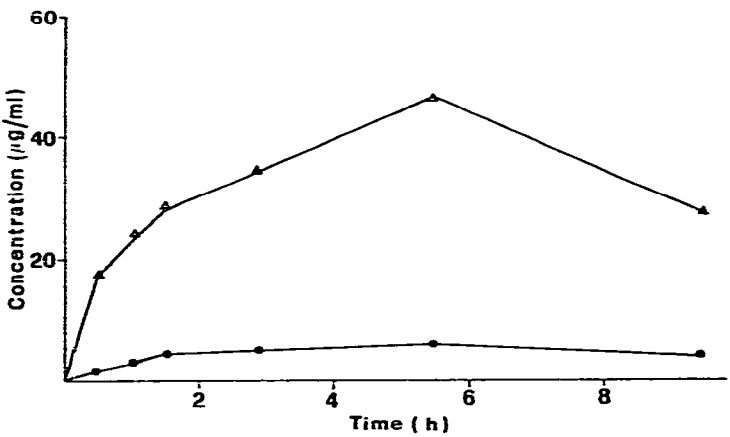

Fig. 3. Serum time course of misonidazole (△) and desmethylmisonidazole (●) for a patient with a bladder tumour after oral administration of 0.6 g/m² of misonidazole.

An application of the proposed TLC method is demonstrated in Fig. 3, which shows the time course of misonidazole and desmethylmisonidazole for a patient with a bladder tumour receiving 0.6 g/m² of misonidazole orally.

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	Differential-pulse polarography				
Detection limit (μg/ml)	$E_{1/2}$** vs. SCE (mV)	$E'_{1/2}$***	$E''_{1/2}$***	Resolution	Detection limit (μg/ml)
≈0.5	—620	—690	—950	poor	≈0.1
≈0.2	—640	—700	—965		≈0.1

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