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SIMULTANEOUS DETERMINATION OF MISONIDAZOLE AND DESMETHYLMISONIDAZOLE IN PLASMA BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

A high-performance liquid chromatographic method for the simultaneous determination of misonidazole and desmethylmisonidazole in plasma is described. After plasma is deproteinized with methanol and the diluted supernatant is chromatographed on a C_{18} reversed-phase column, both compounds are quantitated by means of an internal standard. The coefficients of variation of within-day and day-to-day precision are below 5.0% for misonidazole in the concentration range of 25—250 mg/l and below 6.1% for desmethylmisonidazole in the concentration range of 2.5—25.0 mg/l. Calibration curves are linear and an analytical recovery varying from 97.6 to 99.8% is obtained. The detection limits for misonidazole and desmethylmisonidazole in plasma are 1.4 mg/l and 0.7 mg/l, respectively.

INTRODUCTION

Misonidazole, 1-(2-nitroimidazol-1-yl)-3-methoxypropan-2-ol (Fig. 1, I), has been shown to increase the sensitivity of hypoxic cells to the effects of ionizing radiation in vitro [1]. This so-called radiosensitizing effect of misonidazole could also be demonstrated in vivo in various experimental tumours in animals [2, 3] and in human tumours [4, 5].

The radiation enhancement ratio of hypoxic cells increases with the concentration of misonidazole in the cells [1]. Consequently, to obtain the greatest benefit of the treatment of patients with tumours by a combination of radiotherapy and administration of misonidazole it is desirable to attain levels of misonidazole as high as possible during irradiation. However, the administration of misonidazole to man is limited by its neurotoxicity. Side-effects and neurotoxic symptoms [6—12] have been reported in several studies of the administration of misonidazole to humans. The incidence of neurotoxic symptoms

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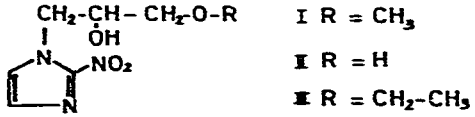

Fig. 1. Chemical structures of misonidazole (I), desmethylmisonidazole (II) and the internal standard (III).

seemed to be related to the total dose of misonidazole administered in a certain period [5—8, 11, 13, 14]. In order to perform toxicological and pharmacokinetic studies of misonidazole in man it was necessary to develop a rapid, sensitive and specific method for the determination of misonidazole and its O-demethylated metabolite, desmethylmisonidazole (Fig. 1, II), in biological material.

Several methods for the determination of misonidazole have been published. Previously reported UV spectrophotometric procedures [10, 15, 16] are not specific for misonidazole, because these methods do not differentiate between the parent compound and desmethylmisonidazole, which has an almost identical UV-absorption spectrum. Polarographic procedures [16, 17] also lack specificity, because all drug-related species with the intact nitroimidazole nucleus are determined simultaneously.

Although paper (PC) [18], thin-layer (TLC) [19] and gas—liquid chromatography (GLC) [16, 19] do separate misonidazole and desmethylmisonidazole, these separation techniques are not optimal. PC and TLC procedures are inadequate for pharmacokinetic studies, because they lack sensitivity and accuracy. GLC methodology requires a rather tedious extraction and derivatization procedure and is unsuitable for the analysis of large numbers of samples usually encountered in pharmacokinetic studies. A high-performance liquid chromatographic (HPLC) method [20] involving protein precipitation by methanol, centrifugation and injection of the methanolic supernatant shows serious problems. Column contamination probably due to accumulation of plasma proteins led to a continuously increasing column pressure. Moreover, even small injection volumes of 10 μl produced peak distortion, which became more of a problem if larger volumes were injected. Another HPLC method [21] lacks the use of an internal standard and also the supernatant is injected directly. Therefore, a rapid, sensitive and specific procedure for the simultaneous determination of misonidazole and desmethylmisonidazole in plasma has been developed. The procedure involves a simple protein precipitation followed by aqueous dilution of the supernatant obtained after centrifugation. Then misonidazole and desmethylmisonidazole are separated on a microparticulate reversed-phase column and detected by monitoring the UV absorbance of the column effluent.

EXPERIMENTAL

Chemicals and reagents

Misonidazole [1-(2-nitroimidazol-1-yl)-3-methoxypropan-2-ol (Ro 07-0582)], desmethylmisonidazole [1-(2-nitroimidazol-1-yl)-2,3-propandiol (Ro 05-9963)] and the internal standard [1-(2-nitroimidazol-1-yl)-3-ethoxypropan-2-ol (Ro 07-0913)] (Fig. 1, III) were kindly provided by Hoffmann-La Roche

& Co. (Mijdrecht, The Netherlands). Analytical reagent grade methanol (Merck, Darmstadt, G.F.R.) and double-distilled water were used. Both solvents were filtered through a 0.45-μm filter before use.

Apparatus

The high-performance liquid chromatograph consisted of a Model 995 isochromatographic pump coupled to a Model 970A variable-wavelength UV—Vis absorbance detector with auto scan option (both from Tracor Instruments, Austin, TX, U.S.A.). Injections were achieved with a Model 7120 syringe loading sample injector (Rheodyne, Berkeley, CA, U.S.A.). This six-port rotary valve was fitted with a 100-μl sample loop. Loading of the sample loop was accomplished with a low-pressure syringe (Scientific Glass Engineering, Ringwood, Australia).

Separations were performed on a reversed-phase column (25 cm × 4.6 mm I.D.) constructed of stainless steel and prepacked with a C_{18} hydrocarbon stationary phase, chemically bonded to silica gel with a mean particle size of 10 μm (LiChrosorb 10 RP-18, from Chrompack, Middelburg, The Netherlands). Detector output was recorded at 1 mV on a flat-bed recorder (Technicorder F Type 3052, Yokogawa Electric Works, Tokyo, Japan).

A Fi-streem 4-litre bidistillation unit (Fisons Scientific Apparatus, Loughborough, Great Britain) was used to prepare double-distilled water. An all-glass filter apparatus with appropriate 0.45-μm filters (Solvent Clarification Kit, Waters Associates, Milford, MA, U.S.A.) was always used to filter solvents before use. An ultrasonic bath was employed to degass the mobile phase before use. Other equipment included a vortex-type mixer, reciprocating shaker, high-speed centrifuge and 100 × 16.25 mm disposable glass tubes with polypropylene caps.

Chromatographic conditions

Chromatography was performed at ambient temperature. The mobile phase, consisting of a methanol—water mixture (20:80, v/v), was delivered at a constant flow-rate of 2.0 ml/min. The resulting operating pressure was approximately 9.65 MPa (1400 p.s.i.). Detection is made by monitoring the UV absorbance of the column effluent at 323 nm. The sensitivity of detection was 0.010 absorbance unit full-scale deflection (AUFS). The detector output was recorded at 1 mV and the chart speed of the recorder was 20 cm/h.

Standards

Aqueous stock solutions of desmethylmisonidazole (250 mg/l) and of misonidazole (2500 mg/l) and a methanolic stock solution of the internal standard (220 mg/l) were prepared. Working standard solutions were made by appropriate dilution of the stock solutions with water or methanol.

Plasma standards containing both misonidazole and desmethylmisonidazole were prepared by adding 9 ml of drug-free plasma to 1 ml of aqueous working standard solutions containing both drugs at various concentrations. Concentrations in the plasma standards ranged from 2.5 to 25.0 mg/l for desmethylmisonidazole and from 25 to 250 mg/l for misonidazole. The standard solutions were stored in the dark at 4°C and were stable for at least two months.

Procedure

Add 9 ml of methanolic internal standard solution (22.0 mg/l) to 1 ml of plasma or working standard solution in a glass tube. Extract the mixture on a reciprocating shaker for 5 min and centrifuge at 2000 *g* for 10 min. After centrifugation transfer 1 ml of the supernatant to a second glass tube and dilute with 4 ml of bidistilled water. Vortex for 30 sec and inject an aliquot into the high-performance liquid chromatograph.

Quantitation

Plasma standards were always included to calculate unknown plasma concentrations of misonidazole and desmethylmisonidazole. Peak height ratios of the drug or its metabolite to the internal standard were calculated. The respective calibration curves were constructed and the best fitting line was found using a least-squares linear regression method. All chromatographic results reported were the mean of duplicate or triplicate injections of every sample or solution, unless otherwise stated.

RESULTS AND DISCUSSION

Chromatographic system

A chromatographic system of a reversed-phase column and a polar mobile phase was preferred, because in such a system polar compounds including drug metabolites are eluted quickly.

Methanol—water mixtures were tested as mobile phases. Volume ratio and

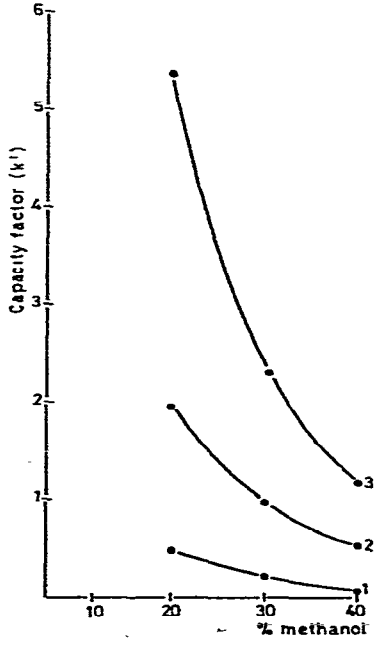

Fig. 2. Plot of capacity factors (*k'*) of some nitroimidazoles against methanol content of the mobile phase. Unretained compound: methanol. Flow-rate: 2.0 ml/min. Chromatographic conditions as described. 1 = Desmethylmisonidazole; 2 = misonidazole; 3 = internal standard.

flow-rate of the methanol—water mixture were varied to achieve optimum chromatographic conditions. The influence of the percentage of methanol in the mobile phase on the capacity factors of the respective nitroimidazoles is shown in Fig. 2. A methanol—water mixture (20:80, v/v) at a flow-rate of 2.0 ml/min was found to be optimum, because separation and resolution of the three nitroimidazoles was achieved adequately. The UV absorption spectra of the three nitroimidazoles in the proposed mobile phase all show absorption maxima at 223 nm and 323 nm. We preferred 323 nm as wavelength of detection, because at this wavelength the highest absolute peak heights were obtained. Under the conditions of this chromatographic system the three nitroimidazoles were eluted as sharp and symmetrical peaks, allowing use of peak heights to quantitate detector response. Retention times for desmethylmisonidazole, misonidazole and internal standard were 2.0, 4.0 and 8.6 min, respectively. The chromatograms of a plasma blank (A), a standard solution in eluent (B) and a plasma standard (C) are shown in Fig. 3. A chromatogram of a plasma sample of a patient with carcinoma of the cervix 5.5 h after oral intake of 0.8 g/m^2 misonidazole is shown in Fig. 4.

Procedure

In order to obtain complete protein precipitation 9 ml of methanol were added to 1 ml of plasma. When the undiluted methanolic supernatant of a plasma standard containing 100 mg/l misonidazole and 10 mg/l desmethylmisoni-

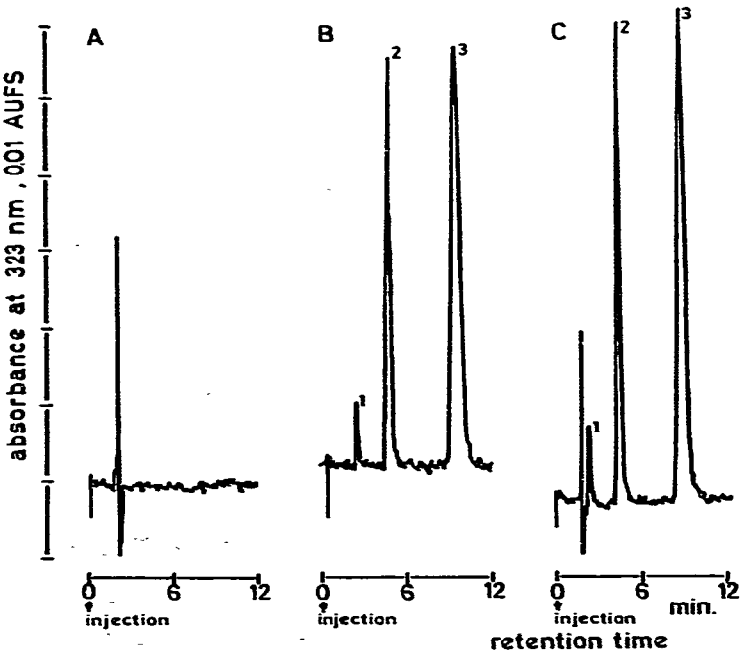

Fig. 3. (A) Chromatogram of blank plasma. (B) Chromatogram of a chromatographic standard solution containing 0.2 mg/l desmethylmisonidazole, 2 mg/l misonidazole and 4 mg/l internal standard. (C) Chromatogram of a plasma standard containing 10 mg/l desmethylmisonidazole and 100 mg/l misonidazole. Chromatographic conditions as described. Injection volume: 75 µl. Peaks: 1 = desmethylmisonidazole; 2 = misonidazole; 3 = internal standard.

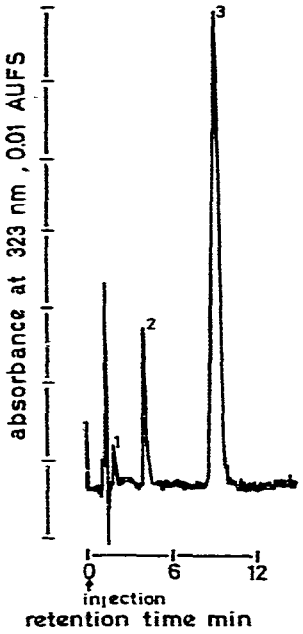

Fig. 4. Chromatogram of a plasma sample of a patient with carcinoma of the cervix 5.5 h after oral intake of 0.8 g/m² misonidazole. Chromatographic conditions as described. Injection volume: 75 µl. Peaks: 1 = desmethylmisonidazole; 2 = misonidazole; 3 = internal standard.

dazole was injected, it became evident that even at a small injection volume of 10 µl the peak shape was not ideal and peak heights were low, although peak height ratios were correct. However, on further increase of the injection volume the peaks became increasingly distorted and at injection volumes greater than 50 µl even peak height ratios became low. After aqueous dilution of the methanolic supernatant of the plasma standard, distortion of the peaks became less as the volume ratio of methanol and water in the injected dilution approximated more closely to that in the mobile phase. Only supernatants, that were diluted with a three- to ninefold volume of water, showed correct peak shape, peak heights and peak height ratios. Therefore, we may conclude that it is not recommended to inject the methanolic supernatant of a plasma sample directly as described previously [20].

Precision

Precision was always evaluated with four pools of plasma containing 25, 50, 100 and 150 mg/l misonidazole and 2.5, 5.0, 10.0 and 15.0 mg/l desmethylmisonidazole. After processing 1-ml aliquots of the plasma pools according to the described procedure and injecting the resulting solutions, precision was calculated as the coefficient of variation of the peak height ratio of misonidazole or desmethylmisonidazole to the internal standard.

The precision of the chromatographic process from injection to detection was determined by making eight identical, successive injections of the resulting solution from one sample of each pool of plasma. The precision of the whole

method was determined by establishing the within-day and day-to-day precision. Within-day precision was determined by processing eight aliquots of 1 ml from each plasma pool and injecting the resulting solutions the same day. Day-to-day precision was established by analyzing one 1-ml aliquot of each plasma pool on each working day during a two-week period. Data on precision of the chromatographic process, within-day and day-to-day precision are presented in Table I.

TABLE I

PRECISION DATA OF THE PROPOSED METHOD FOR THE DETERMINATION OF NITROIMIDAZOLES IN PLASMA

	Plasma conc. (mg/l)	Coefficient of variation (%)		
		Chromatographic process (n = 8)	Within-day (n = 8)	Day-to-day (n = 10)
Desmethylmisonidazole	2.5	5.9	4.5	6.1
	5.0	3.6	4.1	5.4
	10.0	4.3	3.5	4.7
	15.0	2.6	1.5	2.3
Misonidazole	25	0.9	1.9	5.0
	50	1.1	0.6	3.2
	100	1.3	0.7	2.5
	150	0.7	1.2	3.3

Recovery

The results of the recovery study summarized in Table II demonstrate complete recovery of the nitroimidazoles from plasma at the indicated plasma concentrations. The recovery data represent the average value of four extractions at each concentration.

Linearity

The calibration curves for misonidazole and desmethylmisonidazole showed good linearity over the respective concentration ranges 25—250 mg/l and 2.5—25.0 mg/l both in water and in plasma. Plasma levels of misonidazole and desmethylmisonidazole in patients after oral intake of therapeutic doses of misonidazole are reported to be in the concentration ranges mentioned above [6, 22, 23]. The data for the calibration curves of both drugs are summarized in Table III.

Sensitivity and detection limit

Misonidazole and desmethylmisonidazole may be determined at the highest detector sensitivity because the described procedure yields an almost clean extract. The lower limits of detection measured at a detector sensitivity of 0.005 AUFS, with a detector noise of 1×10^{-4} absorbance unit and allowing a signal-to-noise ratio of 2, are 2.0 ng and 1.0 ng for misonidazole and desmethylmisonidazole, respectively.

TABLE II

ANALYTICAL RECOVERY OF NITROIMIDAZOLES FROM PLASMA

	Plasma conc. (mg/l)	Recovery* (%)
Desmethylmisonidazole	2.5	97.6
	5.0	98.1
	10.0	99.4
	15.0	98.7
Misonidazole	25	98.4
	50	99.8
	100	99.0
	150	99.3
Internal standard	50	99.2
	100	98.8
	200	99.5
	300	99.8

*Means of four determinations at each concentration.

TABLE III

DATA FOR THE CALIBRATION CURVES OF DESMETHYLMISONIDAZOLE AND MISONIDAZOLE IN PLASMA

	Concentration (mg/l)	Peak height ratio*		Linear regression parameters**		
		Mean	C.V. (%)	Slope	*y*-intercept	Correlation coefficient
Desmethyl-misonidazole	2.5	0.039	5.4	0.016	−0.004	0.9998
	5.0	0.075	4.2			
	10.0	0.157	1.1			
	15.0	0.239	1.3			
	20.0	0.326	2.3			
	25.0	0.402	0.2			
Misonidazole	25	0.248	2.2	0.010	−0.010	0.9999
	50	0.488	1.3			
	100	0.987	0.9			
	150	1.483	0.9			
	200	1.986	0.3			
	250	2.500	0.9			

*Data represent mean and coefficient of variation (C.V.) of three replicate injections at each concentration.

**Peak height ratio of drug to internal standard plotted on the *y*-axis versus drug concentration in plasma (in mg/l) on the *x*-axis.

Analyzing a 1-ml plasma sample according to the procedure of this method and injecting 75 μl of the resulting solution the lower limits of quantification are 1.4 mg/l and 0.7 mg/l for misonidazole and desmethylmisonidazole, respectively.

Clinical application

The method described has been applied to the determination of plasma levels of misonidazole and desmethylmisonidazole in patients with various tumours receiving therapeutic doses of misonidazole in combination with radiotherapy. The results of these clinical studies will be reported soon.

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