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Note

Determination of itraconazole in plasma and animal tissues by high-performance liquid chromatography

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Itraconazole, (±)-*cis*-4-[4-[4-[4-[[2-(2,4-dichlorophenyl)-2-(1*H*-1,2,4-triazol-1-ylmethyl)-1,3-dioxolan-4-yl]methoxy]phenyl]-1-piperazinyl] phenyl]-2,4-dihydro-2-(1-methylpropyl)-3*H*-1,2,4-triazol-3-one (I, Fig. 1) is a new, orally active triazole antifungal [1,2].

In order to study the pharmacokinetics of the compound in humans and animals, a sensitive and specific assay method for the determination of the drug in biological samples was needed. Because of the physicochemical properties of the compound, a high-performance liquid chromatographic (HPLC) method was selected.

EXPERIMENTAL

Standards and reagents

Itraconazole (I, R 51 211) and the internal standard (II, R 51 012), *cis*-4-[4-[4-[4-[[2-(2,4-dichlorophenyl)-2-(1*H*-1,2,4-triazol-1-ylmethyl)-1,3-dioxolan-4-yl]methoxy]phenyl]-1-piperazinyl]phenyl]-2,4-dihydro-5-methyl-2-(3-methylbutyl)-3*H*-1,2,4-triazol-3-one (Fig. 1) were obtained as reference compounds from the Janssen Life Sciences Products Division (Beerse, Belgium).

Spectrophotometric-grade acetonitrile, *n*-heptane and methanol were used; the other solvents were of analytical grade and included diethylamine and isoamyl alcohol. The inorganic reagents were prepared in doubly distilled water.

Stock solutions, corresponding to 0.1 mg/ml methanol, were prepared for itraconazole and the internal standard. Standard solutions were obtained by diluting the itraconazole stock solution to concentrations down to 0.020 μg/ml. The inter-

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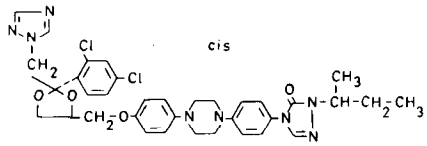
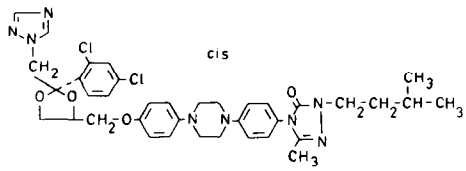


I



II

Fig. 1. Chemical structures of itraconazole (I) and the internal standard (II).

nal standard stock solution was diluted to final concentrations of 2 and 10 μg/ml.

Apparatus

All chromatographic analyses were performed on a Hewlett-Packard Model 1084B high-performance liquid chromatograph, equipped with a variable-volume injector (79841 A), an automatic sampling system (79842 A) and a variable-wavelength detector (79875 A) operating at 263 nm or a fixed-wavelength detector (79870 A) operating at 254 nm. The separations were achieved using a reversed-phase 15 cm × 2.1 mm I.D. column, packed with 5-μm octadecyl particles (RSiL C18HL, Alltech Europe) by the balanced density procedure by means of an air-driven fluid pump (Haskel). The samples were eluted with water—acetonitrile (40:60) at a constant flow-rate of 0.5 ml/min. To suppress the ionization of the basic functions of the investigated compounds, 0.05% diethylamine was added to the solvent system. Area integrations, calculations and plotting of the chromatograms were carried out by a Hewlett-Packard 79850 LC terminal.

Extraction procedure

Plasma samples (1 or 2 ml; unknowns, drug-free plasma or standards containing known amounts of itraconazole), spiked with the internal standard (1 or 0.2 μg), were buffered to pH 7.8 with a 0.05 *M* phosphate buffer and extracted twice for 10 min with 4 ml of heptane—isoamyl alcohol (98.5:1.5, v/v). The organic phase was back-extracted with 3 ml of 0.05 *M* sulphuric acid and removed after centrifugation (1000 *g*). The remaining acidic phase was made alkaline with concentrated ammonia (pH 9) and re-extracted twice with 2-ml aliquots of the same heptane—isoamyl alcohol mixture. The combined organic layers were then evaporated to dryness under a gentle stream of nitrogen in a water-bath at 55 °C and submitted to HPLC analysis.

Animal tissues, ground in a Waring commercial blender, were homogenized (1:4, w/v) in distilled water using an Ultra-Turrax homogenizer. Then 1- or 2-

ml aliquots of the homogenates were submitted to the plasma extraction procedure.

The various extraction residues were redissolved in 100 μl of the elution solvent, and 40-μl aliquots were injected onto the HPLC column.

Calibration procedure

Using the standard solutions, samples of blank plasma or tissue homogenate (1 or 2 ml) were spiked with itraconazole at concentrations ranging from 1 ng/ml to 10 μg/ml, and with the internal standard at fixed concentrations of 0.1 μg/ml (lower-concentrated samples) or 1 μg/ml (higher-concentrated samples). All calibration samples were taken through the extraction procedures described above.

Calculations

Ultimate sample concentrations were calculated by determining the peak-area ratio of itraconazole relative to the internal standard, and comparing this ratio with the standard curve, obtained after analysis of the calibration samples. For quantification of lower-concentrated samples, peak-height ratios were also used.

RESULTS AND DISCUSSION

The extractability of itraconazole, a very lipophilic, weak organic base, from plasma and animal tissues was tested in recovery experiments using different alkaline buffer systems with heptane—isoamyl alcohol as the extraction solvent. The optimum compromise between extraction recovery and chromatographic purity was obtained at pH 7.8 using heptane—isoamyl alcohol (98.5:1.5, v/v). Over the whole concentration range studied, the recovery amounted to $71.5 \pm 2.3\%$ (mean $\pm$ S.D., $n = 11$) for plasma samples. For various spiked tissue homogenates (1 μg/ml), the extraction recovery ranged from 64 to 73%.

Fig. 2 shows chromatograms of blank human plasma (A), control plasma spiked with itraconazole (B) and plasma from a volunteer after intake of itraconazole (C). The internal standard concentration was 100 ng/ml for all three samples. Chromatograms of extracts of plasma from rats are depicted in Fig. 3. All samples were spiked with an internal standard concentration of 1 μg/ml. The chromatogram in Fig. 3C was obtained after analysis of plasma from a rat, 96 h after termination of a repeated oral dosing schedule of 40 mg/kg per day for four weeks. As can be seen, no interfering peaks were encountered at the retention times of itraconazole (4.3 min) or its internal standard (5.8 min), and both compounds eluted as completely resolved peaks.

Linear relationships were found when the peak-area or peak-height ratios of itraconazole to the internal standard were plotted versus the itraconazole plasma concentrations. The standard curves, analysed over a six-month period, gave the mean regression parameters summarized in Table I. A separate standard curve was run for each series of samples.

The accuracy and reproducibility of the method were ascertained by replicate analyses of quality-control samples, spiked with 100 or 1000 ng/ml internal standard and calculated using either peak-area integration or manual measurements of peak heights. The data obtained from peak-height measurements are given in

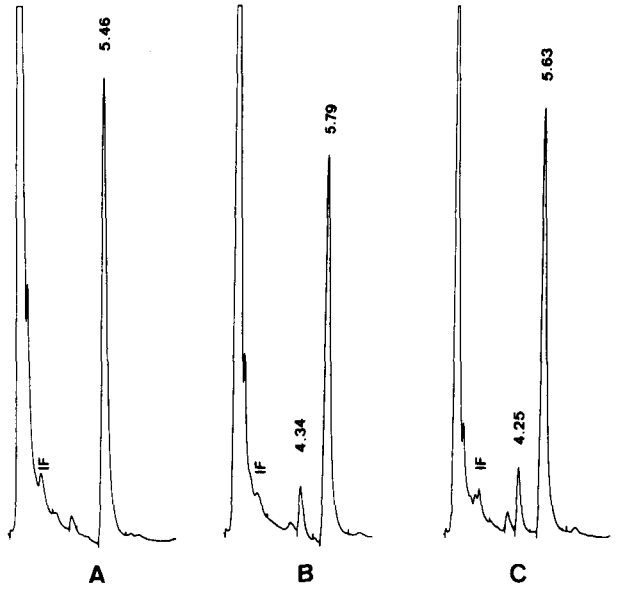


Fig. 2. Chromatograms of extracts from (A) blank control plasma spiked with 100 ng/ml internal standard (retention time, $t_R = 5.46$ min); (B) control plasma spiked with 10 ng/ml itraconazole ($t_R = 4.34$ min) and 100 ng/ml internal standard ($t_R = 5.79$ min); (C) plasma from a volunteer, 24 h after oral intake of 100 mg of itraconazole as a capsule, containing 12.1 ng/ml itraconazole ($t_R = 4.25$ min) and 100 ng/ml internal standard ($t_R = 5.63$ min). IF is a special integrator symbol.

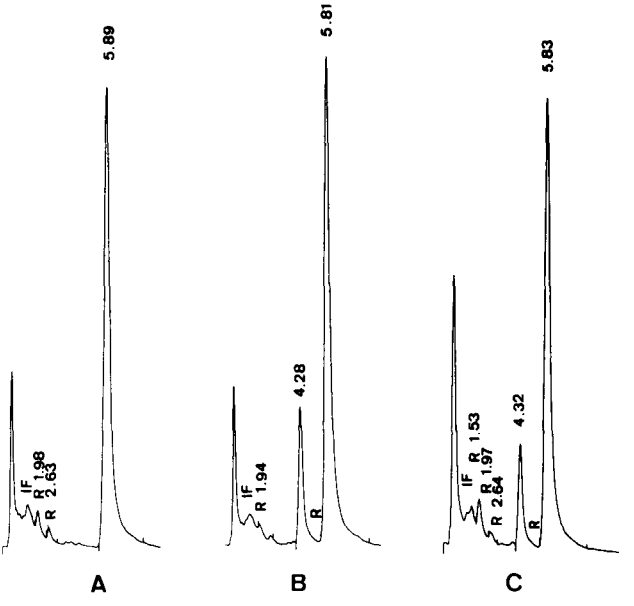


Fig. 3. Chromatograms of extracts from (A) blank control plasma, spiked with 1 μg/ml internal standard ($t_R = 5.89$ min); (B) control plasma spiked with 0.200 μg/ml itraconazole ($t_R = 4.28$ min) and 1 μg/ml internal standard ($t_R = 5.81$ min); (C) plasma from a rat, 96 h after termination of a repeated oral dosing schedule of 40 mg/kg per day for four weeks, containing 0.169 μg/ml itraconazole ($t_R = 4.32$ min) and 1 μg/ml internal standard ($t_R = 5.83$ min). IF and R are special integrator symbols.

TABLE I

MEAN STANDARD CURVES FOR ITRACONAZOLE IN PLASMA

Calculation method	Internal standard concentration (μg/ml)	Range (μg/ml)	Regression equation ($y=ax+b$)★		Correlation coefficient (r)	n
			a	b		
Peak area measurements	1.0	0.050–10.0	1.262	−0.026	0.9999	14
	0.1	0.005–1.00	13.29	−0.030	0.9999	10
Peak-height measurements	1.0	0.005–0.500	1.571	−0.005	0.9995	15
	0.1	0.001–0.100	14.91	−0.00002	0.9999	8

★y = peak-area or peak-height ratio (itraconazole to internal standard); x = itraconazole plasma concentration (μg/ml).

TABLE II

ACCURACY AND REPRODUCIBILITY OF THE HPLC METHOD FOR THE DETERMINATION OF ITRACONAZOLE IN PLASMA SAMPLES

Itraconazole added (ng/ml)	Internal standard concentration (ng/ml)	Itraconazole found (mean ± S.D., $n=6$) (ng/ml)	Coefficient of variation (%)	Relative error (%)
1.0	100	1.1 ± 0.2	13.9	+9.0
2.5	100	2.5 ± 0.2	8.5	0.0
5	100	4.8 ± 0.1	2.5	−4.7
10	100	9.5 ± 0.7	7.1	−5.3
25	100	25.1 ± 1.2	4.7	+0.5
50	1000	50.1 ± 2.2	4.5	+1.0
100	1000	99.6 ± 0.7	0.7	−0.4
250	1000	250 ± 5.6	2.2	−0.1
500	1000	501 ± 1.7	0.3	+0.2

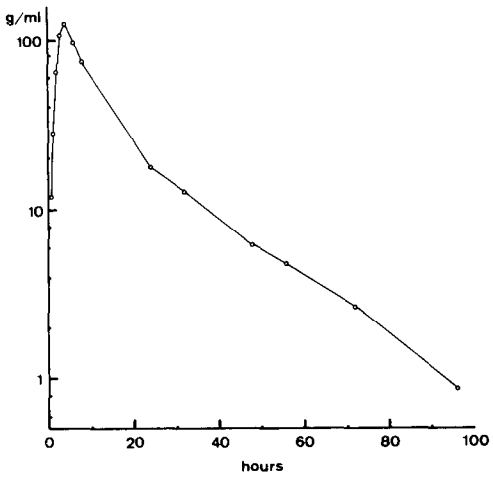


Fig. 4. Mean plasma levels of itraconazole in six volunteers after oral administration of 100 mg.

Table II. Reliable results are obtained, even at the lower limit of detection, which appeared to be ca. 1 ng/ml. Peak-area measurements gave comparable results, although, because of the inadequacy of the integration system used, accurate estimation of plasma levels below 10 ng/ml mostly failed.

The method described has been used to study the pharmacokinetics in laboratory animals and the comparative bioavailability of different oral formulations in humans. The plasma concentration—time profile in a group of six healthy volunteers after intake of 100 mg of itraconazole as two 50-mg PEG capsules is depicted in Fig. 4. A mean elimination half-life of 17.1 h was found.

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