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Note

Measurement of ketoconazole, a new antifungal agent, by high-performance liquid chromatography

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Ketoconazole (*cis*-1-acetyl-4-[4-[[2-(2,4-dichlorophenyl)-2-(1H-imidazol-1-ylmethyl)-1,3-dioxolan-4-yl] methoxy] phenyl] piperazine) is an antifungal agent of the imidazole type which is effective after oral administration against superficial and systemic fungal infections [1, 2]. At present, several microbiological assay procedures have been developed for measuring the concentration of drug in plasma [1, 3—5]. These methods determine unknown concentrations of ketoconazole in plasma by measuring growth inhibition of a specific microorganism by a given volume of plasma. This inhibition is then compared against the inhibition produced by known standards of ketoconazole. The major potential problem with these methods is that they detect total antifungal activity in plasma and may not, therefore, be specific for ketoconazole alone. Two high-performance liquid chromatographic (HPLC) procedures for determining concentrations of ketoconazole in plasma have been described [6, 7]. Both methods employ UV detection at low wavelengths. Hence, time-consuming extraction procedures are used to decrease the amounts of endogenous compounds in plasma which might potentially interfere with the determination of the drug. In addition, neither method employs an internal standard to facilitate quantitation of ketoconazole concentrations in plasma.

The present study describes an HPLC procedure with fluorescence detection for measuring concentrations of ketoconazole in plasma. The method employs an internal standard and is sufficiently rapid and sensitive for use in pharmacokinetic studies. Data comparing the bioassay [5] with the HPLC procedure which we have developed are shown.

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EXPERIMENTAL

Standards and solvents

Standard solutions of ketoconazole (R41400) and the internal standard (R41300) (*cis*-ethyl-4-[4-[[2-(2,4-dichlorophenyl)-2-(1H-imidazol-1-ylmethyl)-1,3-dioxolan-4-yl] methoxy] phenyl] -1-piperazine carboxylate) (both gifts of Janssen Pharmaceutica, Beerse, Belgium) are made in methanol. Standard concentrations of ketoconazole ranged from 0.10 to 2.5 μg per 100 μl methanol. The concentration of the internal standard solution was 1.0 μg per 50 μl methanol. New solutions of both compounds were made every two weeks and stored in foil-wrapped containers at 4°C.

Unless specified, all organic liquids are distilled-in-glass (Burdick and Jackson Labs., Muskegon, MI, U.S.A.). In addition, the diethyl ether used in the extraction is initially washed as follows: for 1 l diethyl ether, wash two times with approximately 50 ml 2 *N* sodium hydroxide, three times with 50 ml 0.1 *N* sulfuric acid, and four times with 100 ml glass-distilled water. Aqueous reagents are prepared in glass-distilled water.

Extraction

The extraction procedure involves a simple alkaline extraction into diethyl ether, evaporation and reconstitution with methanol. A 50-μl aliquot of the internal standard solution is pipetted into a culture tube (13 × 100 mm) with a PTFE-lined screw cap. Next, 0.50—2.0 ml plasma are added and alkalinized with 200 μl 2 *N* sodium hydroxide. Three ml of washed diethyl ether are then added to the tube, and the mixture is allowed to shake on a Labquake shaker (Labindustries, Berkeley, CA, U.S.A.) for 10 min, followed by centrifugation at a relative centrifugal force of 1100 *g* for 5 min. The tube is immersed in a dry ice—acetone bath and the aqueous phase is frozen. The ether phase is then poured into a 15-ml conical centrifuge tube and evaporated to dryness under vacuum in a vortex evaporator (Buchler Instruments, Fort Lee, NJ, U.S.A.) at 40°C. The residue is reconstituted with 50 μl of methanol. A 25-μl aliquot is injected into the chromatograph.

Chromatographic conditions

A Varian Model 8500 high-pressure liquid chromatograph (Varian Instruments, Palo Alto, CA, U.S.A.) fitted with a Rheodyne loop injector (Rheodyne, Cotati, CA, U.S.A.) and a μBondapak C_{18} reversed-phase column, 30 cm × 3.9 mm, 10 μm (Waters Assoc., Milford, MA, U.S.A.) are used for the separation. The mobile phase mixture, 60% acetonitrile and 40% 0.05 *M* Sorensen's phosphate buffer (pH 6.6 with hydrochloric acid) is filtered through a Whatman No. 2 filter and degassed before use. The flow-rate is 90 ml/h.

Detector

A Schoeffel FS970 fluorescence detector (Schoeffel Instruments, Westwood, NJ, U.S.A.) with quartz cell and a deuterium lamp is used. The excitation monochromator is set at 206 nm and a 370-nm cutoff emission filter is used. The output from the detector is recorded on a Varian Model A-25 recorder.

Calibration

The assay is calibrated by analyzing 1-ml aliquots of control plasma to which have been added 0.10—2.5 μg of ketoconazole and 1.0 μg of internal standard. For each sample, the ratio of ketoconazole peak height to internal standard peak height is determined. Each ratio is divided by the amount of ketoconazole in that sample to give a normalized peak height ratio. The peak height ratios are plotted against the concentrations to determine the linearity of the extraction and detector response. The normalized peak height ratios are averaged, and the mean value is used to determine the amounts of ketoconazole in unknown samples. Precision of the assay is estimated by determining the coefficient of variation of the normalized peak height ratios.

RESULTS AND DISCUSSION

Retention times under the conditions described are 5.2 min for ketoconazole and 9.7 min for the internal standard. One sample can be chromatographed in 11—12 min. Fig. 1 shows chromatograms of extracts from 1.0 ml control plasma and 1.0 ml plasma containing 0.25 μg ketoconazole and 1.0 μg internal standard. The two compounds are well separated, and there are no significant peaks which interfere with the peaks of interest.

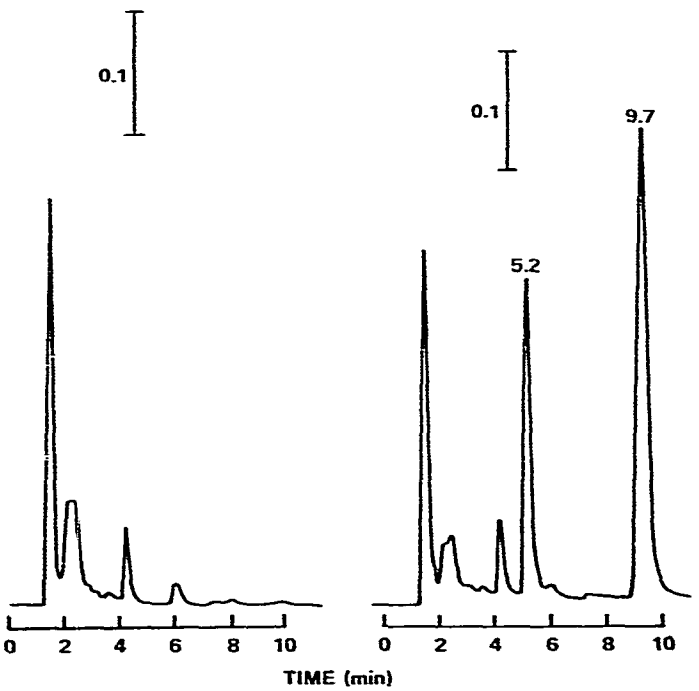

Fig. 1. Chromatograms of extracts from blank human plasma (left) and blank plasma spiked with 0.25 μg of ketoconazole (retention time 5.2 min) and 1.0 μg of internal standard (retention time 9.7 min) (right).

Reproducibility

The reproducibility of the assay was measured by determining the coefficients of variation of five replicate extractions of 0.10, 0.25, 0.50, and 1.0 μg ketoconazole in 1 ml plasma. Reproducibility with varying amounts of plasma was determined by extracting 0.25 μg ketoconazole from 0.50, 1.0, 1.5, and 2.0 ml plasma. Table I shows coefficients of variation for the calibration graphs and the reproducibility studies. These results indicate that the overall variability of the assay is low. The calibration graph is linear and passes through the origin. The reproducibility studies at different concentrations show low variability, even at 0.10 μg/ml. Reproducibility with different volumes is somewhat less than the reproducibility obtained with equal volumes. The lower limit of sensitivity for the assay is 100 ng extracted from plasma. This is sufficient sensitivity for use in pharmacokinetic studies of the drug, as will be seen in the study below.

TABLE I

SUMMARY OF DATA FOR CALIBRATION GRAPHS AND REPRODUCIBILITY STUDIES

	Ketoconazole (μg)	Sample size (ml)	n*	Coefficient of variation (%)
Calibration graph	0.10—2.5	1	10	6.3
Reproducibility	0.10	1	5	6.1
	0.25	1	5	4.3
	0.50	1	5	3.5
	1.0	1	5	3.2
	0.25**	0.50—2.0	16	12.4

*n = number of samples.

**Reproducibility with varying volumes.

Recovery

The recovery of ketoconazole from the extraction procedure was determined by extracting 1 ml of plasma containing either 0.25 or 1.0 μg of unlabelled ketoconazole together with a tracer (1400 dpm) of [^{3}H]ketoconazole (specific activity = 390 Ci/mg, Janssen Pharmaceutica) and counting a 25-μl aliquot of the final extract of ketoconazole in 50 μl of methanol. The average recoveries are 74 and 79% after addition of 0.25 and 1.0 μg of ketoconazole, respectively.

Specificity

Because we did not have any potential metabolites of ketoconazole, specificity of the assay for ketoconazole is not known. One metabolic pathway involves initial scission of the imidazole ring [8]. Other likely metabolic pathways are via hydrolysis of the various ether functions, and the metabolites thus formed would probably not interfere with ketoconazole due to large differences in their chemical structure. Miconazole, another antifungal agent, did not interfere with the determination of ketoconazole.

Comparison with the bioassay

Plasma samples from patients receiving ketoconazole as a single dose or during chronic administration of ketoconazole were assayed by both the HPLC procedure and the bioassay procedure [5]. A graph of these data is shown in Fig. 2. The comparison indicates that although there is a general trend for the

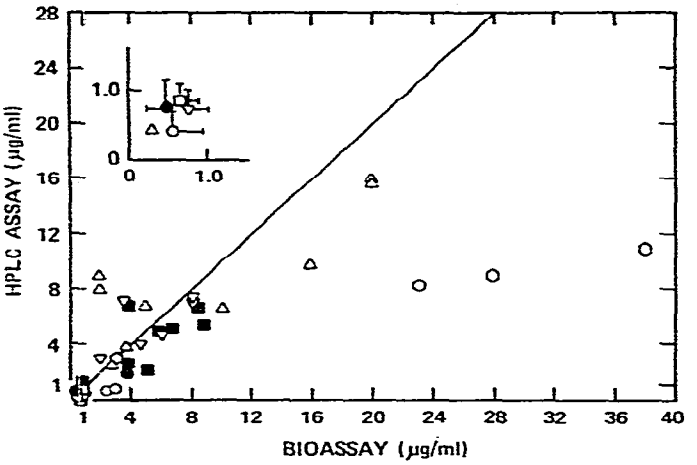

Fig. 2. Relationship between plasma concentrations of ketoconazole obtained by the HPLC and the bioassay procedure. Each symbol represents the plasma concentrations obtained in a particular individual. The open symbols represent samples obtained after the first dose of ketoconazole, while the closed symbols represent plasma concentrations obtained after chronic treatment in the same individual. The line is the line of identity. For clarity, concentrations that are lower than 1.0 μg/ml by both assay procedures are shown in the inset and where appropriate, plotted as the mean ± standard deviation.

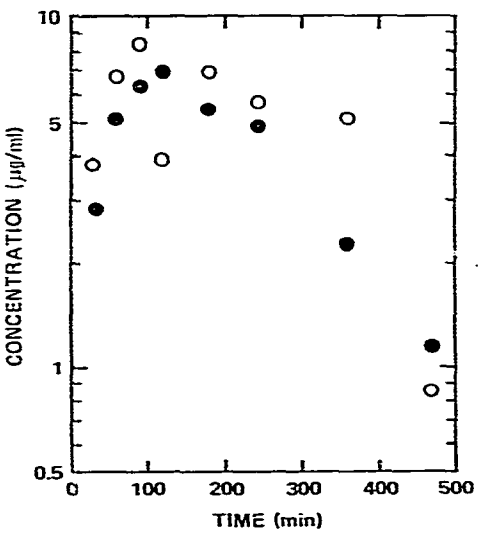

Fig. 3. Concentrations of ketoconazole in plasma obtained from a single subject who received 200 mg ketoconazole orally. (•) represents concentrations obtained by analysis with the HPLC procedure and (○) represents concentrations obtained by analysis with the bioassay.

two procedures to give similar results, there is no concordance. The discordant results are most apparent at high concentrations, where there is nonlinearity of the zone size (plotted as radius of inhibition zone minus radius of well, squared) versus drug concentration plot in the bioassay [9]. The lack of concordance between the HPLC assay and the bioassay was observed after single doses of ketoconazole and after administration of multiple doses. Fig. 3 illustrates the plasma concentrations of ketoconazole obtained by both procedures in a single subject at various times after he received ketoconazole orally. The curve obtained by the chromatographic procedure is smooth while that obtained by the bioassay is somewhat erratic.

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