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DETERMINATION OF THE ANTIFUNGAL AGENT, KETOCONAZOLE, IN HUMAN PLASMA BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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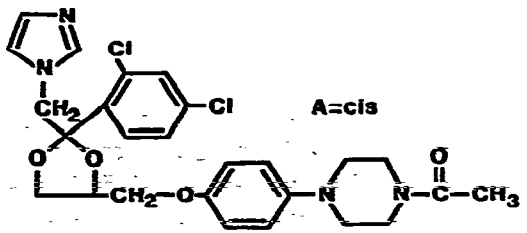
SUMMARY

A rapid and selective high-performance liquid chromatographic (HPLC) assay for the quantitative determination of ketoconazole, an orally active antifungal agent, in human plasma is described. After extraction of the drug from plasma, the compound is separated by HPLC using a reversed-phase column and detected by UV light at 205 nm. Quantitation is accomplished by external standardization and the determination of peak areas is performed with the aid of an integrating computer. The average recovery of ketoconazole over a concentration range of 0.1—20.0 μg/ml was 88.2 ± 4.07% S.D. The maximum sensitivity of the assay is less than 0.1 μg/ml. The assay is suitable for use in pharmacokinetic studies following the administration of therapeutic doses of ketoconazole to humans.

INTRODUCTION

Ketoconazole (I; *cis*-1-acetyl-4-[4-[[2-(2,4-dichlorophenyl)-2-(1H-imidazol-1-ylmethyl)-1,3-dioxolan-4-yl]methoxy]phenyl]piperazine) (Fig. 1) is an



(I)

Fig. 1. Structure of ketoconazole (I).

orally active broad-spectrum antifungal agent [1] effective in vivo and in vitro against different forms of candidiasis [2], dermatophytosis [3], and coccidioidomycosis [4] in man and other animal species. A microbiological assay [2,4]

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has been employed for the determination of ketoconazole in plasma. The plasma levels, as measured by the bioassay, varied between 1 and 6 μg/ml in man following the oral administration of 200 mg of ketoconazole. The microbiological assay, however, is not specific since it determines the total antifungal activity of the plasma which might also include a contribution due to the presence of active metabolites. This paper describes a new high-performance liquid chromatographic (HPLC) method for the determination of ketoconazole in human plasma which is rapid, selective and suitable for use in pharmacokinetic studies. No interferences from three potential metabolites of ketoconazole nor from miconazole (Monistat I.V.®), which could be administered concomitantly with this drug [5,6], were observed. Although a gas–liquid chromatographic assay has been used for the analysis of biological specimens [7], this represents the first published assay for ketoconazole capable of determining levels of unchanged drug in plasma following the administration of this potent antimycotic imidazole.

EXPERIMENTAL

Apparatus

Analyses were performed on a liquid chromatographic system composed of a Waters Model M6000A pump (Waters Assoc., Milford, MA, U.S.A.) equipped with a LC-55B variable-wavelength UV (205 nm) detector (Perkin-Elmer, Norwalk, CT, U.S.A.). Separations were accomplished at ambient temperature on a 30 cm × 3.9 mm I.D. μBondapak/CN reversed-phase column (Waters Assoc.). Samples were introduced onto the column through a Model U6K septumless injector (Waters Assoc.) with a 25-μl Pressure-Lok syringe (Precision Sampling, Baton Rouge, LA, U.S.A.). Chromatograms were traced on a Linear Model 300 (Linear Instruments, Irvine, CA, U.S.A.) strip-chart recorder (1 mV) and peak area integration was performed by a Hewlett-Packard (Avondale, PA, U.S.A.) 3354B integrating computer interfaced with the detector by employing a Model 18652A (Hewlett-Packard) A/D converter. Analog signal modification to reduce noise from the UV detector to both the strip chart recorder and A/D converter was achieved with the use of a Model 1021A electronic filter and amplifier (Spectrum Scientific, Newark, DE, U.S.A.).

Reagents and solvents

Ketoconazole (I), three potential metabolites of ketoconazole: *cis*-1-acetyl-4-(4-hydroxyphenyl)piperazine (II); *cis*-1-acetyl-4-[4-(1,2-dihydroxyethyl)methoxyphenyl] piperazine (III); and *cis*-1-[4-[[2-(2,4-dichlorophenyl)-2-(1H-imidazol-1-ylmethyl)-1,3-dioxalan-4-yl] methoxy] phenyl] piperazine (IV); three structural analogs of ketoconazole: *cis*-1-[4-[[2-(2,4-dichlorophenyl)-2-(1H-imidazol-1-ylmethyl)-1,3-dioxolan-4-yl] methoxy] phenyl] -4-(1-methylethyl)-piperazine trihydrochloride 2-propanolate (V), *cis*-methyl-4-[4-[[2-(2,4-dichlorophenyl)-2-(1H-imidazol-1-ylmethyl)-1,3-dioxolan-4-yl] methoxy] -phenyl] -1-piperazine acetate (VI), *cis*-ethyl-4-[4-[[2-(2,4-dichlorophenyl)-2-(1H-imidazol-1-ylmethyl)-1,3-dioxolan-4-yl] methoxy] phenyl] -1-piperazine carboxylate dihydrochloride hemihydrate (VII), and miconazole (1-[2,4-dichloro-β-(2,4-dichloro-benzyloxy)phenethyl] imidazole) (VIII), were synthe-

sized and supplied by Janssen Pharmaceutica (Beerse, Belgium). All chemicals and reagents except acetonitrile, methanol, and ethyl acetate (distilled in glass, Burdick & Jackson, Muskegon, MI, U.S.A.) were reagent grade.

Chromatographic conditions

The mobile phase was composed of 0.05 *M* KH_2PO_4—NaOH buffer (pH 6.0)—acetonitrile (65:35, v/v) and the flow-rate adjusted to 2.0 ml/min. The solvent mixture was prepared daily using doubly distilled water and degassed under reduced pressure before use.

Instrument settings

The output voltage (1 V/2 a.u.f.s.) of the LC-55B spectrophotometer was adjusted using a signal attenuator (Perkin Elmer) set at 0.2 a.u.f.s. and the time constant set at the "normal" position. Noise suppression of the spectrophotometric output to both the recorder and the A/D converter was achieved by adjusting the cutoff frequency of the Model 1021A Electronic filter to 0.01 Hz and the gain of the amplifier to ×1. The net output to the recorder (1 mV) under these conditions (i.e. the sensitivity of the detector) was determined to be 0.01 a.u.f.s.

Extraction

An aliquot (2.0 ml) of plasma was transferred to a 15-ml test-tube (15 × 125 mm) fitted with a PTFE-lined screw cap, alkalinized by the addition of ammonium hydroxide (0.25 ml), and then extracted twice with ethyl acetate (5.0 ml) by vigorously shaking the mixture for 30 sec. Separation of the layers was facilitated by centrifugation for 1 min at 1500 *g*. The organic layers were transferred with the aid of a Pasteur pipette and pooled in a clean screw-cap test-tube. Drug was back extracted into sodium chloride-saturated 1 *N* HCl (2.0 ml), the phases separated by centrifugation as before, and the organic layer discarded. The aqueous phase was extracted again with ethyl acetate (5.0 ml) and the organic layer discarded. Final extraction of the drug with ethyl acetate (twice with 5.0 ml) was accomplished after alkalinization of the aqueous phase with ammonium hydroxide (0.5 ml). The phases were separated after each extraction by centrifugation to obtain a clear supernatant, and the organic layers were transferred and pooled in a 20-ml glass vial. An aliquot (1.0 ml) of 1% ammoniacal methanol was added to the ethyl acetate extract before concentrating the sample to dryness under a stream of nitrogen in a water-bath (50°C). The resulting residue was reconstituted and quantitatively transferred with 1% ammoniacal methanol (ca. 2 ml) to a 2.5-ml conical centrifuge tube. The contents of the conical tube were concentrated into the tip by washing the walls of the tube several times with aliquots (ca. 0.2 ml) of 1% amoniacal methanol. The final residue was dissolved in the HPLC mobile phase before separation by HPLC. All samples were analyzed the same day they were extracted.

Extraction efficiency

Blood from several untreated volunteers was drawn into heparinized vacutainers® and centrifuged to generate a plasma pool from which an extraction

efficiency was established using the following procedure. Known amounts of ketoconazole (I), dissolved in methanol (0.01, 0.1 and 1.0 μg/μl), were added to aliquots (2.0 ml) of drug-free plasma, achieving concentrations of 0.1, 0.5, 2.0, 5.0, 10.0 and 20.0 μg/ml. Replicate samples ($n>6$) in each concentration group were thoroughly mixed after fortification with drug, and stored frozen (−20°C) in screw-cap test-tubes for one week until extracted as previously described. All samples at each concentration were analyzed in duplicate. Since the prepared drug concentrations ranged over two orders of magnitude (0.10—20.0 μg/ml), varying volumes of the final residue dissolved in the HPLC mobile phase were injected for analysis by HPLC. Typically, when the drug concentration approached 0.1 μg/ml, one-fifth (20 μl/100 μl) of the dissolved residue volume was analyzed, whereas at a concentration of 20.0 μg/ml only one-four hundredth (5 μl/2000 μl) of the dissolved residue was injected. After separation by HPLC, quantitation of ketoconazole was accomplished by use of the external standard method described below.

Calibration and standard solution preparation

Ketoconazole (25.0 mg) was dissolved in methanol and diluted to volume in a 25-ml volumetric flask to achieve a concentration of 1.0 μg/μl. Solutions containing 0.1 and 0.01 μg/μl of ketoconazole were prepared daily by serial dilution of the 1.0 μg/μl methanolic stock solution with the mobile phase system described earlier. Solutions of compounds II—VIII were prepared by dissolving a quantity (10 mg) of each standard in methanol (10 ml) to achieve a concentration of about 1 μg/μl.

The external standard method, prepared from the software section of the computer, is predicated on a linear relationship between amount injected and peak area (μV-sec as reported by the integrating computer). This relationship was initially evaluated by repeated ($n\geqslant4$) injections of 20, 50, 100, 150 and 200 ng of ketoconazole from the 0.01 μg/μl stock solution prepared with mobile phase. Thereafter, an average calibration response factor was established by the computer following triplicate injections of 100 ng of ketoconazole.

Specificity

Assay specificity was evaluated by injecting aliquots (1 μl) of the prepared methanolic solutions containing either ketoconazole (I), its potential metabolites (II, III, IV), structural derivatives of ketoconazole (V, VI, VII) or miconazole nitrate (VIII) onto the HPLC column to determine their relative separation. In addition, a portion of the extract (20 μl/100 μl) from a drug-free plasma sample was also routinely evaluated at the highest sensitivity (0.01 a.u.f.s.) for the presence of any extractable UV-absorbing (205 nm) material which might interfere with the measurement of ketoconazole.

RESULTS AND DISCUSSION

The average recovery of drug from plasma samples, to which ketoconazole had been added, was determined to be 88.2 ± 4.07% S.D. (Table I) with an average precision (C.V.) of 4.61%. Statistical analysis [8] demonstrated that there were no significant differences ($p > 0.227$) among the mean recoveries

TABLE I

RECOVERY OF KETOCONAZOLE FROM HUMAN PLASMA AT VARIOUS CONCENTRATIONS

Theoretical plasma concentration (μg/ml)	Average observed plasma concentration (μg/ml)	*n*	Average recovery* (%)
0.1	0.0876	16	87.6 ± 5.06
0.5	0.448	5	89.6 ± 8.52
2.0	1.714	7	85.7 ± 2.89
5.0	4.435	6	88.7 ± 1.24
10.0	8.90	6	89.0 ± 2.13
20.0	17.66	5	88.3 ± 4.57
			$\bar{x}$ 88.2 ± 4.07%**

*$\bar{x}$ ± S.D.
**C.V. = 4.61%.

from each concentration group. These data, therefore, suggest that there is no concentration dependence on extraction efficiency over the range of drug plasma levels which was evaluated.

Linear regression analysis of the curve described by plotting peak area (μV-sec) versus ng injected indicated a linear fit of the data ($p > 0.05$) from 0 to 200 ng with a coefficient of determination (r^2) equal to 0.9975. The slope of the line was calculated to be 1268.5 μV-sec/ng and the intercept, which was not significantly different from zero ($p < 0.05$), determined to be 2962.6 μV-sec. Over a two-month period of analysis, the slope of this line demonstrated little change, with a coefficient of variation equal to 2.6%.

The capacity factors (k') for ketoconazole (I) and compounds II—VIII are listed in Table II. Baseline separation between ketoconazole and the three potential metabolites (compounds II—IV) is achieved within 11 min. Two of the structural analogs (compounds VI and VII) elute with retention times similar to that observed for ketoconazole and one of its potential metabolites (IV) and would not be appropriate as internal standards under the chromatographic conditions described. The third analog, compound V, has a retention time that would require a total analysis time in excess of 20 min. The presence of miconazole (VIII) in a plasma sample at therapeutic concentrations, as a

TABLE II

CAPACITY FACTORS (k') OF KETOCONAZOLE AND COMPOUNDS II—VIII AS DETERMINED BY HPLC

Compound	k'
I	4.18
II	0.85
III	0.53
IV	7.47
V	12.0
VI	4.65
VII	7.23
VIII	10.2

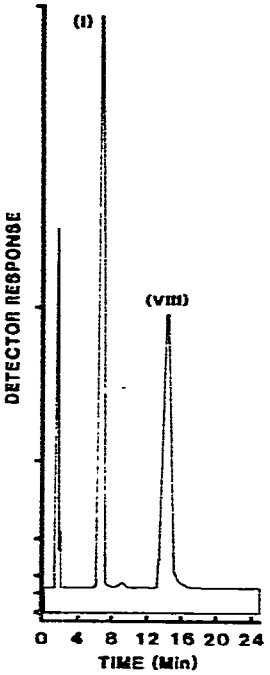

Fig. 2. HPLC chromatogram of ketoconazole (I) and miconazole (VIII). Approximately 1 μg of each compound was injected and detected at 205 nm (0.05 a.u.f.s.).

result of concomitant therapy with Monistat I.V.® [5,6], would not interfere with the measurement of ketoconazole (Fig. 2).

After separation by HPLC, ketoconazole was detected at 205 nm ($\epsilon \approx 24{,}750$ in ethanol). Absolute amounts of 0.01 μg of ketoconazole can be determined at a sensitivity of 0.01 a.u.f.s. However, since there are a large number of potential chromophores such as co-extracted endogenous compounds or other impurities that are active at 205 nm, it is important that the highest quality solvents be utilized for both the extraction of plasma and the preparation of mobile phase. Otherwise, undesirably high detector background might interfere with the measurement of ketoconazole. Ethyl acetate extracts from drug-free plasma were free of interfering UV (205 nm) absorbing peaks (Fig. 3A). On occasion, when a peak with a retention time similar to that of ketoconazole was observed in a drug-free plasma extract, it was found to be equivalent to less than 0.01 μg/ml. A representative chromatogram of extract from plasma containing ketoconazole concentrations of 0.1 μg/ml is shown in Fig. 3B. Samples from quality control pools containing known amounts of ketoconazole have been stored frozen (—20°C) for up to one week without decomposition.

CONCLUSIONS

In summary, an HPLC assay for the determination of ketoconazole in plasma has been developed which is suitable for use in pharmacokinetic studies following the administration of therapeutic doses to humans. The method is rapid and specific for unchanged drug and is capable of measuring plasma levels as low as

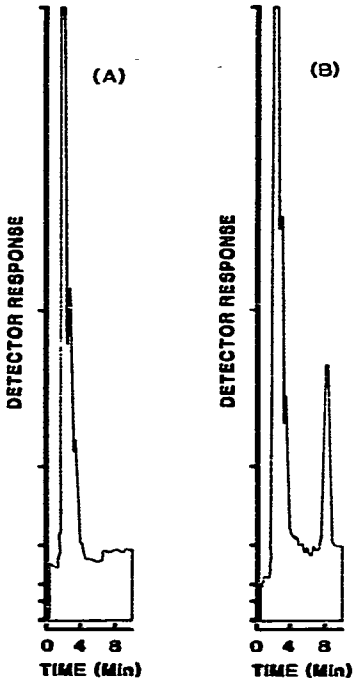

Fig. 3. Representative chromatograms of extracts from (A) drug-free plasma; one-fifth of the total extract volume (100 μl) dissolved in the HPLC mobile phase was injected and analyzed at UV (205 nm); (B) human plasma containing 0.1 μg/ml of ketoconazole. Detector response for both chromatograms is 0.01 a.u.f.s.

0.1 μg/ml. Data from this study have demonstrated that both recovery and detection of ketoconazole are linear over the range of clinically significant concentrations of this drug in human plasma.

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