The thermogram for liposomes containing II–III (60:40 mole %) shows a transition peak with a T_c value of 48° (Fig. 4a). This value is intermediate to the T_c values of pure II (57°) and pure III (41.5°), indicating good bilayer mixing. The thermograms after incubation with either Ca^{2+} or neomycin, or both (Fig. 4b, c, and d), are similar to those of liposomes composed of I–III, suggesting that the chain length of the neutral lipid does not drastically influence the overall nature of these interactions.

The displacement of calcium, which has been described for the interaction of the drug with bacteria (19), the neuromuscular junction (20), and inner ear tissues (21), may interfere with normal calcium-dependent physiological functions. The results of this study indicate that these effects may be due, in part, to resultant changes in the thermotropic properties, *i.e.*, degree of fluidity, of the lipid bilayer induced by neomycin.

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Quantitation of Ketoconazole in Biological Fluids Using High-Performance Liquid Chromatography

V. L. PASCUCCI * ˣ, J. BENNETT ‡, P. K. NARANG *, and D. C. CHATTERJI * ¶

Received May 5, 1982, from the **Clinical Pharmacokinetics Research Laboratory, Department of Pharmacy, The Clinical Center, National Institutes of Health, Bethesda, MD 20205* and the ‡*Laboratory of Clinical Investigation, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, MD 20205.* Accepted for publication November 2, 1982. ¶Present address: Lyphomed Inc., Melrose Park, IL 60160.

Abstract □ A rapid, specific procedure is described for the quantitation of ketoconazole in biological fluids using high-performance liquid chromatography (HPLC). The procedure involves sample preparation using a reverse-phase C-18 cartridge prior to chromatography and quantitation using peak height ratios (UV absorbance detection, 231 nm) of ketoconazole to the internal standard, phenothiazine. A sensitivity of 0.2 μg/ml was achieved using a 0.5-ml sample. The mean recovery was 86.2%, and overall coefficient of variation of the procedure was 7.1%. This procedure has been used to determine ketoconazole levels in human serum, plasma, CSF, and synovial fluid. A comparison with a microbiological assay is presented, and adaptability of this procedure to quantitation by fluorescence to increase the sensitivity fivefold is discussed.

Keyphrases □ Ketoconazole—quantitation in biological fluids, high-performance liquid chromatography, humans □ High-performance liquid chromatography—quantitation of ketoconazole in biological fluids, humans

Ketoconazole, *cis*-1-acetyl-4-[4[[2-(2,4-dichlorophenyl)-2- (1*H*-imidazol-1-ylmethyl) -1,3-dioxolan-4 - yl] methoxy]phenyl]piperazine, an antifungal agent used to treat a wide variety of superficial and systemic mycoses (1–3), has the advantage over other imidazole derivatives of producing adequate, sustained blood levels following oral administration. Several microbiological assays (4–6) and three high-performance liquid chromatographic (HPLC) methods (7–9) have been described for quantitation of ketoconazole in biological fluids. HPLC techniques have the advantage of direct concentration measurement since the microbiological assays quantitate ketoconazole concentration indirectly as antifungal activity against a test organism. Microbiological procedures lack specificity for ketoconazole; thus, falsely elevated levels may be produced by active metabolites or other concurrently administered antifungal agents.

A rapid and reproducible HPLC method is described which is suitable for quantitation of ketoconazole in biological fluids both for routine monitoring (sensitivity to 0.2 μg/ml using UV detection) or for pharmacokinetic studies

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(sensitivity to 40 ng/ml using fluorescence detection). Comparison of the results obtained using the described method with the values obtained using a microbiological assay (6) is also presented.

EXPERIMENTAL

Materials and Reagents—Ketoconazole[1], (R 41,400), was used in the preparation of spiked serum standards. Phenothiazine[2] was used as the internal standard. Methanol[3] was glass distilled and certified HPLC grade. Water was double-distilled in glass. All HPLC solvents were filtered[4] and then deaerated under reduced pressure prior to use. Reverse-phase C-18 sample cartridges[5] were used in sample preparation.

Chromatographic System and Conditions—The liquid chromatograph was equipped with a UV absorbance detector[6] operated at 231 nm and a solvent delivery system[7]. Alternatively, a spectrofluorometric detector[8] was operated with an excitation wavelength of 206 nm and a 370-nm emission filter. The analytical column[9] (4.6 mm i.d. × 25 cm, 5-μm particle size) was preceded by a 7-cm guard column[10]. The mobile phase consisted of of 75% (v/v) methanol and 25% (v/v) 0.02 *M* monobasic sodium phosphate. The pH of the mobile phase was adjusted to 6.8 with sodium hydroxide. The flow rate was maintained at 1.0 ml/min.

Procedures—Deproteinization and elimination of polar serum constituents were accomplished using C-18 reverse-phase sample preparation cartridges. The cartridges were conditioned by washing with 2.0 ml of methanol followed by 5.0 ml of water. A 0.5-ml aliquot of serum sample or serum standard was placed in a 1.5-ml capped plastic tube[11]. Phenothiazine, 20 μl of a 25-μg/ml solution (for UV detection) or 30 μl of a 100-μg/ml solution (for fluorescence detection), was added to each sample to serve as an internal standard. An alkaline pH was achieved with the addition of 0.125 ml of 0.1 *N* NaOH to each sample. The samples were mixed by inversion, and each was added to a reverse-phase cartridge. Each cartridge was then washed with 6.0 ml of water followed by 2.0 ml of methanol. The eluant from the final 1.5 ml of methanol was collected in a disposable 12- × 75-mm borosilicate culture tube[12] and evaporated to dryness using a gentle air stream at 40°. The residue was reconstituted with 250 μl of methanol–water (1:1) and mixed by vortexing. A 50-μl aliquot was injected onto the HPLC.

Ketoconazole serum standards were prepared by spiking human serum with ketoconazole stock solution (100 μg/ml in methanol) to give final concentrations ranging from 0.5 to 15.0 μg/ml. Ketoconazole was quantitated by comparison of the peak height ratio of the drug to the internal standard using a calibration curve. The peak height ratios were plotted against concentrations of ketoconazole and analyzed by linear regression to generate daily calibration curves. The lower sensitivity limit of the procedure was determined by assaying spiked serum samples at concentrations ranging from 20 ng/ml to 1.0 μg/ml. The sensitivity limit was then defined as the concentration at which the signal-to-noise ratio was 3.

Interday variability was determined by the reproducibility of the daily standard curves ($n \geq 6$) with respect to both their slopes and the calculated values for ketoconazole at various concentrations (0.5, 1.0, 2.0, 5.0, 10.0, and 15.0 μg/ml). The intraday variability was assessed by performing replicate analyses ($N = 6$) using spiked serum samples containing 0.8 and 14.0 μg/ml of ketoconazole. These concentrations were chosen as a representation of the lower (0.8 μg/ml) and higher (14.0 μg/ml) serum concentrations observed in patients receiving ketoconazole for whom routine therapeutic drug monitoring was performed. Aqueous solutions containing known amounts of ketoconazole were compared with spiked serum standards undergoing analysis to calculate the percent recovery at various concentrations. Statistical analyses to determine means, standard deviations, correlation coefficients, coefficients of variation, and linear regressions were performed using a computer[13].

The microbiological assay procedure was performed as described by Drouhet and Dupont (6). The test organism was *Kluyvermyces fragilis* strain 55-1[14].

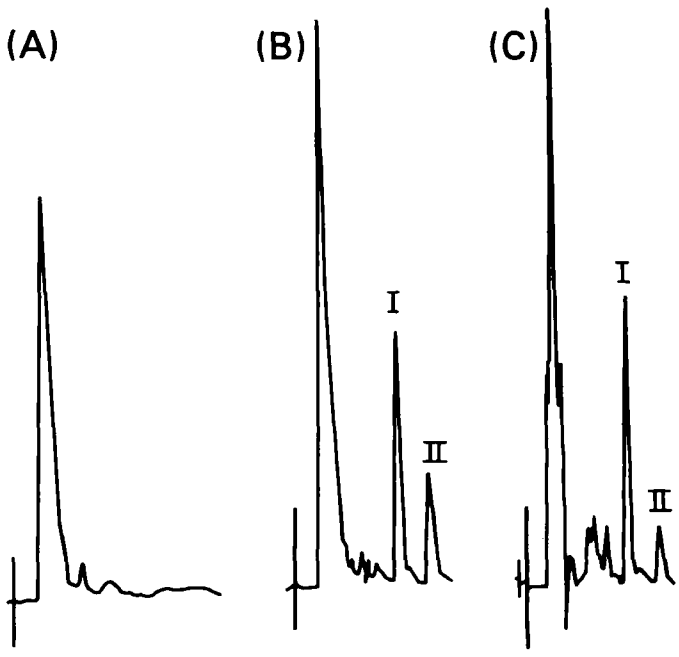

Figure 1—*Representative chromatograms of (A) blank human serum, (B) serum obtained from a patient receiving ketoconazole and spiked with phenothiazine at 0.04 AUFS (observed ketoconazole concentration = 1.13 μg/ml), and (C) human serum spiked with 0.50 μg/ml of ketoconazole (retention time 9.6 min) and phenothiazine (retention time 7.4 min) at 0.04 AUFS. Sample preparation was as described in the text. Key: (I) phenothiazine (internal standard); (II) ketoconazole.*

RESULTS AND DISCUSSION

HPLC Using UV Detection—Representative chromatograms from assayed samples of control human serum, human serum spiked with phenothiazine and ketoconazole, and a serum sample obtained from a patient receiving ketoconazole are shown in Fig. 1. Retention times for phenothiazine and ketoconazole are 7.4 and 9.6 min, respectively. No interfering peaks were observed in the blank serum sample. Miconazole, another imidazole antifungal agent, as well as trifluoperazine, chlorpromazine, diazepam, chlordiazepoxide, doxepin, thiothixene, imipramine, and amitriptyline did not interfere with either the ketoconazole or internal standard peaks. The reverse-phase cartridge separation procedure described helped eliminate several additional peaks which were observed when the serum samples were extracted using various organic solvents.

The standard calibration curves constructed from daily runs of spiked serum standards were linear and highly reproducible. Serum standards containing up to 25 μg/ml have been assayed periodically, and the assay results indicate that the linearity of the curves extends at least to this level. The mean slope of six calibration curves run over a 3-month period was 0.456, with a standard deviation of 0.032; the overall coefficient of variation of the procedure was 7.1%. The correlation coefficient of the six curves was 0.998. Table I lists the results of the interday variability (mean, *SD*, *CV*) from the six runs at each concentration measured. These data indicate the assay procedure is highly reliable and reproducible.

Intraday variability of the method determined from serum standards containing 0.8 and 14.0 μg/ml of ketoconazole was 6.4 and 4.4%, respectively. The average recovery of ketoconazole obtained from the sample preparations over the range of concentrations used in the standard curve was 86.2% (*SD* 4.6%). The recovery of phenothiazine was 91.0% (*SD* 6.4%). Using the described procedure, 0.2-μg/ml concentrations of ketoconazole can be detected while maintaining a signal-to-noise ratio of 3.

[1] Ketoconazole was a gift of Janssen Pharmaceutica, New Brunswick, N.J.
[2] Aldrich Chemical Co., Milwaukee, Wis.
[3] Burdick and Jackson Laboratories, Muskegon, Mich.
[4] 0.45-μm Millipore filter; Millipore Corp., Bedford, Mass.
[5] Waters Associates, Inc., Milford, Mass.
[6] Schoeffel model GM770; Schoeffel Instrument Corp., Westwood, N.J.
[7] Spectra-Physics model 3500B; Spectra-Physics, Santa Clara, Calif.
[8] Schoeffel model FS970; Schoeffel Instrument Corp., Westwood, N.J.
[9] Altex Ultrasphere Octadecylsilane; Beckman Instruments, Inc., Berkeley, Calif.
[10] Guard column packed with C-18 (30–38 μm) particles; Whatman, Inc., Clifton, N.J.
[11] Sarstedt, Princeton, N.J.
[12] Fisher Scientific, Silver Spring, Md.
[13] Hewlett-Packard Model 85; Hewlett-Packard, Corvallis, Or.
[14] Test organism provided by Dr. David A. Stevens, Stanford University.

Table 1—Reproducibility Results from Six Replicate Serum Standard Curves Obtained over a 3-Month Period

Spiked Ketoconazole Concentration, μg/ml	Observed Concentration		
	Mean, μg/ml	*SD*	*CV*, %
0.5	0.56	0.09	16.1
1.0	0.98	0.075	7.7
2.0	2.01	0.14	6.9
5.0	4.98	0.18	3.6
10.0	10.04	0.12	1.2
15.0	15.04	0.08	0.53
Overall Mean			7.1

The procedure presented here has significant advantages over two previously published HPLC procedures (7, 8). The major advantage of our method is the use of an internal standard, which facilitates accurate and precise ketoconazole quantitation. Neither of the previous HPLC methods used internal standardization, although they involved either extraction and concentration procedures (7) or a reverse-phase cartridge filtration procedure (8). In addition, the sample preparation of one of the published methods (7) involves multiple time-consuming extraction steps, whereas the other published procedure (8) does not include the statistical analyses necessary for detailed evaluation. Finally, the sensitivity, efficiency, and overall variability of the procedure presented here compare favorably with the previously published HPLC procedures.

This procedure has been used to successfully analyze various biological fluids from patients receiving ketoconazole, including serum, plasma, CSF, and synovial fluid. Serum samples from several patients receiving 200-, 400-, or 800-mg/day doses of ketoconazole were collected at 2 and 6 hr postdose and analyzed using this procedure. It is apparent that the procedure sensitivity is adequate for such routine therapeutic monitoring. When a single 200-mg dose of ketoconazole is administered to adults, peak levels of 3–4.5 μg/ml are observed, and concentrations at 8 hr postdose are at least 0.2 μg/ml (10). On chronic administration, even higher levels are observed. Our own experience indicates that levels in the patients studied at the stated doses range between 1 and 15 μg/ml. For routine serum level monitoring of ketoconazole at therapeutic doses, the present HPLC method using UV detection has been found to be rapid, reliable, and sensitive.

Comparison with a Microbiological Assay—Serum samples from 32 patients were divided and assayed for ketoconazole by both the HPLC method and a microbiological procedure (6). A comparison of the two procedures is shown graphically in Fig. 1, a plot of the results of the microbiological *versus* the HPLC analyses of the patient samples. Linear regression analysis of the comparative data yields a correlation coefficient of 0.92. However, when a line with a slope of unity that passes through the origin is superimposed on the plot (as in Fig. 2), it can be seen that there is a large amount of scatter as well as a negative deviation of the microbiological assay results from the HPLC results at concentrations >10 μg/ml. The scatter can largely be attributed to the imprecision inherent in the bioassay. (This microbiological procedure had a coefficient of variation of 28% for 18 patients' serum samples run 2–5 times for a total of 56 assays.) However, the general agreement of the results of the two assays suggests that the ketoconazole quantitated in patient samples represents a microbiologically active agent.

HPLC Using Fluorometric Detection—Ketoconazole concentrations may be quantitated using the sample preparation and assay conditions described here with a spectrofluorometric detector as well as a UV absorbance detector. There are two advantages of using fluorescence measurement. First, the reverse-phase cartridge separation procedure is not required for routine monitoring of biological fluids. Using a fluorometric detector, a simple acetonitrile protein precipitation followed by centrifugation and direct injection of the supernatant is an adequate sample preparation to achieve sensitivity equivalent to that seen with cartridge filtration and UV absorbance detection. However, by using the reverse-phase cartridge preparation described, the sensitivity of the HPLC procedure can be increased fivefold, detecting <40 ng/ml of ketoconazole using the spectrofluorometric detector. This enhanced sensitivity is an additional advantage over other HPLC methods, including one procedure which also uses fluorometric detection (9). The increase in sensitivity is a distinct advantage for detailed pharmacokinetic studies of ketoconazole disposition where it is likely that the sensitivity requirements will exceed those of all previously published methods. Data obtained using an unpublished GC procedure for ketoconazole indicate that following a dose of ketoconazole, there is an initial rapid decline in serum levels followed by a much slower elimination phase (10). To fully characterize its biphasic elimination, detection of <100 ng/ml of ketoconazole is required.

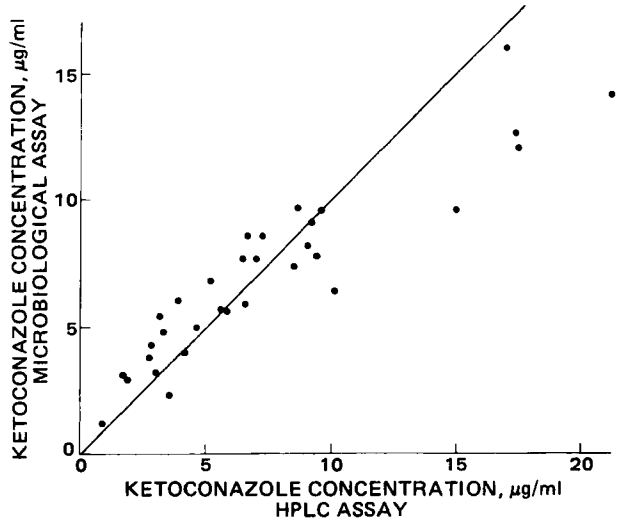

Figure 2—*Comparison of the results of ketoconazole quantitation by microbiological* versus *HPLC analyses. Each point represents parallel determinations of a single patient sample by both methods. The line superimposed on the plot has a slope of unity and passes through the origin.*

In summary, the sensitive, rapid, and reproducible procedure described offers advantages over both microbiological and other HPLC methods. It can be adapted to either UV or fluorometric detectors for routine monitoring of ketoconazole in various biological fluids. Fluorometric detection offers the additional advantage of the increased sensitivity required for application of the procedure to detailed pharmacokinetic studies of ketoconazole disposition.

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