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DETERMINATION OF KETOCONAZOLE IN THE PLASMA, LIVER, LUNG AND ADRENAL OF THE RAT BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

An accurate and precise procedure for the rapid analysis of ketoconazole in the lung, liver, plasma and adrenal gland of the rat has been developed. Separation of the drug from endogenous substances was achieved by solid-phase extraction followed by reversed-phase chromatography on a Novapak C_{18} column using a mobile phase of methanol—acetonitrile—0.02 *M* phosphate buffer (pH 6.8) (35:30:35). Depending on the tissue, the recoveries of ketoconazole and clotrimazole, which was used as an internal standard, ranged between 85.0 to 93.6% and 79.3 to 84.1%, respectively. The reproducibility of the assay was between 3 and 4%, depending on the tissue involved. The sensitivity of the procedure permitted the monitoring of ketoconazole levels for up to 24 h in the adrenal and up to 48 h for plasma, lung and liver, following oral administration of 150 mg/kg of the drug to male rats.

INTRODUCTION

Ketoconazole, *cis*-1-acetyl-4-[4-[[2-(2,4-dichlorophenyl)-2-(1H-imidazol-1-ylmethyl)-1,3-dixolan-4-yl] methoxy] phenyl] piperazine (Fig. 1), is a broad-spectrum antifungal agent structurally related to miconazole. Although the drug is adsorbed orally [1, 2], its bioavailability in humans is influenced by

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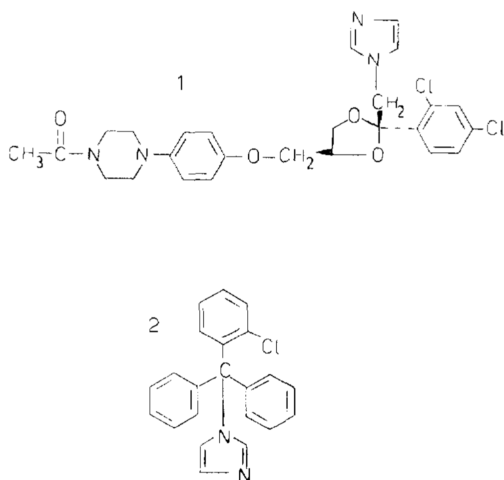

Fig. 1. Structures of ketoconazole (1) and clotrimazole (2).

the acidity of the gastric juice [1] and the presence of food [2]. The present study was prompted by recent observations [3–7] that ketoconazole, like other imidazole-containing drugs, causes changes in cytochrome P-450-dependent monooxygenase activities and in epoxide hydrolase activity. Ketoconazole is a potent inhibitor of cytochrome P-450-dependent steroid hydroxylation in the adrenals [3], and a moderately potent inhibitor of the monooxygenation of drugs and foreign chemicals in liver and lungs [4–7]. In addition, ketoconazole enhances microsomal epoxide hydrolase activity with some epoxide substrates [8]. Assay procedures were required for the determination of ketoconazole in plasma, lung, liver and adrenals of rats so that a relationship between enzyme activity and drug concentration in the tissues can be developed.

Several microbiological methods for the analysis of ketoconazole in plasma have been described [9–12]. However, their lack of specificity has led to the development of a number of procedures which employ high-performance liquid chromatography (HPLC) [13–15]. The first HPLC method for the determination of ketoconazole in plasma was described by Alton [13] and a similar method was published subsequently by Mannisto et al. [2]. Both these methods [2, 13] involved lengthy multiple extractions and did not employ internal standards. A simplified extraction procedure was described later by Swezey et al. [14] who used an analogue of ketoconazole as an internal standard. Subsequently, Pascucci et al. [15] described a simplified sample preparation technique employing C_{18} reversed-phase cartridges and phenothiazine as an internal standard. Recently, Badcock [16] has described a procedure for the therapeutic drug monitoring of ketoconazole in neonates. This procedure [16] uses only 20 μl of serum and involves protein precipitation with acetonitrile, followed by centrifugation and direct injections of the supernatant onto a C_{18} column. None of these procedures [12–16] were found to be directly applicable to the analysis of ketoconazole in the lung, liver and adrenal of the rat and alternative strategies are presented in this study.

EXPERIMENTAL

Apparatus

The liquid chromatograph comprised a Model 110A Beckman pump (Beckman Instruments, Fullerton, CA, U.S.A.), a fixed-wavelength (254 nm) Model 153 Beckman UV detector and a Model 210 Beckman injection valve fitted with a 50-μl loop. The chromatograms were recorded on a Fisher Series 5000 Recordall recorder (Fisher Scientific, Fair Lawn, NJ, U.S.A.). The same Novapak C_{18} column (Waters Assoc., Milford, MA, U.S.A.) (5 μm, 150 × 3.9 mm I.D.) was used throughout.

Chemicals and reagents

Ketoconazole was a gift from Janssen Pharmaceutica (New Brunswick, NJ, U.S.A.). Clotrimazole was obtained from Sigma (St. Louis, MO, U.S.A.). Acetonitrile and methanol were HPLC grade and obtained from Fisher. All the other reagents were reagent grade from various sources. Deionized water was used throughout.

Chromatographic conditions

The column was eluted with a mobile phase of methanol–acetonitrile–0.02 *M* KH_2PO_4/NaOH buffer (pH 6.8) (35:30:35) at a flow-rate of 2.0 ml/min and ambient temperature. Mobile phases were filtered under vacuum and degassed by placing them in a sonicator for 5 min.

Animal studies

Suspensions of ketoconazole in corn oil (75 mg/ml) were administered orally by gavage to male Sprague–Dawley rats (150–200 g) at a dose of 150 mg/kg. Three rats were sampled before administration of the drug and at each of the following times after dosing: 0.5, 1, 2, 4, 8, 24 and 48 h. The rats were anesthetized with diethyl ether and 2–3 ml of blood removed by cardiac puncture and transferred to heparinised tubes. The rats were then sacrificed and the liver, lung and adrenal removed by dissection. The organs were rinsed with 0.15 *M* potassium chloride buffered to pH 7.4 with 0.05 *M* phosphate buffer, and frozen immediately (−20°C). The erythrocytes were separated from the plasma by centrifugation using an International clinical centrifuge (International Equipment, Boston, MA, U.S.A.) operated at full speed (approx. 1500 *g*) for 5 min. The plasma supernatant was then stored at −20°C until analysis.

Sample preparation

For the analysis of ketoconazole in liver, two samples of tissue were weighed (500 mg) and transferred to a Thomas tissue grinder (Series 3431-E04, Arthur H. Thomas, Philadelphia, PA, U.S.A.). A 100-μl volume of a 3 mg/ml methanolic solution of the internal standard (clotrimazole) was pipetted into the grinder, followed by 4 ml of acetonitrile. The tissue was then homogenized for 2 min using a PTFE pestle. The homogenate was centrifuged at full speed for approx. 15 min using the bench top centrifuge described above. The supernatant was removed, transferred to a 16-ml test tube and the solvent

evaporated at 45°C under a gentle stream of nitrogen. The residue was reconstituted with 1 ml of 0.01 *M* hydrochloric acid and adjusted to a pH of approx. 10.5 by the addition of 200 μl ammonium hydroxide. The resultant extract was then applied to the top of a reversed-phase sample preparation cartridge (Spice, Rainin, Woburn, MA, U.S.A.). Before the application of the tissue extract, the sample cartridges were prepared with 2 ml of methanol and 5 ml of water. The adsorbed ketoconazole was washed with 6 ml of water and desorbed with 3 ml of methanol. The methanolic extract was evaporated to dryness at 45°C under a gentle stream of nitrogen, reconstituted in HPLC mobile phase (1 ml) and injected onto the HPLC column. For samples taken at 24 and 48 h, the final residues were reconstituted in 200 μl of mobile phase. Both lungs from each animal were assayed using the same procedure as that used for liver. Typically, each lung weighed about 500 mg and each liver weighed about 10 g.

For the analysis of ketoconazole in plasma, 100 μl of the internal standard solution (3 mg/ml clotrimazole in methanol) and 200 μl of ammonium hydroxide were added to 1 ml of plasma. The basified plasma was then applied directly to a Spice cartridge which was then treated as described above. The final residue was reconstituted in mobile phase (1 ml).

Both the adrenals from each rat were assayed as follows. Each adrenal was weighed (typically 10—20 mg) and transferred to the tissue grinder. Acetonitrile (1 ml) and 100 μl of a 300 μg/ml solution of clotrimazole in methanol were added and the tissue was homogenized for 30 s. The homogenate was centrifuged and treated in the same manner as the lung and liver extracts. The final residue was reconstituted in 200 μl of mobile phase.

Calibration curves

Daily calibration curves were prepared by spiking tissues and plasma from untreated animals; 100, 80, 60, 40 and 20 μl of ketoconazole stock solution (500 μg/ml in 0.01 *M* hydrochloric acid) were added to 1 ml of plasma or 500 mg of lung or liver, followed by 100 μl of internal standard stock solution (3 mg/ml clotrimazole in methanol). For the calibration of the adrenals, complete organs were spiked with ketoconazole and clotrimazole. Single adrenal glands were transferred to tissue grinder tubes and spiked with 100, 80, 60, 40 and 20 μl of a 50 μg/ml stock solution of ketoconazole in 0.01 *M* hydrochloric acid. Acetonitrile (1 ml) and 100 μl of a 300 μg/ml stock solution of clotrimazole were then added to each tube. The spiked tissues were then extracted as described previously.

RESULTS AND DISCUSSION

Using the extraction conditions described, the extracts of lung, liver and plasma were extremely clean and free from endogenous substances which might otherwise interfer with the compounds of interest. Thus ketoconazole and clotrimazole could be eluted from the column with retention times of 4.5 min (k' = 8.0) and 6.8 min (k' = 12.6), respectively, using a simple binary mobile phase of methanol in a phosphate buffer. The overall analysis time was 8.0 min with a mobile phase of 70% methanol in 0.02 *M* phosphate buffer (pH 6.8). However, these chromatographic conditions were unsuitable for the

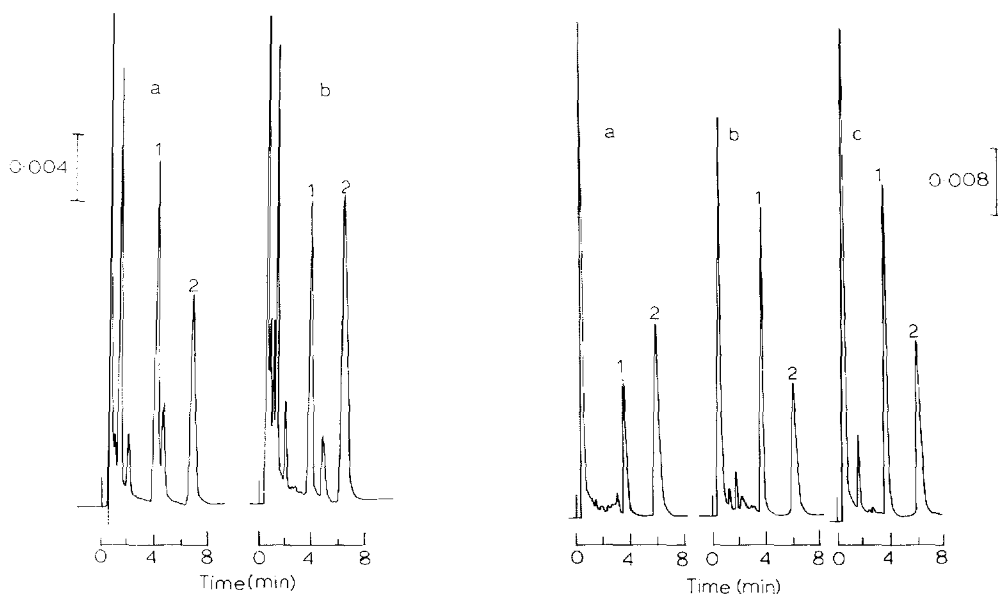

Fig. 2. Effect of mobile phase composition on the separation of ketoconazole from the adrenal of the rat. Stationary phase: Novapak C_{18} (5 μm, 100 mm × 3.9 mm I.D.). Mobile phase: (a) methanol—0.02 *M* phosphate buffer, pH 6.8 (70:30), (b) methanol—acetonitrile—0.02 *M* phosphate buffer, pH 6.8 (35:30:35). Flow-rate: 2.0 ml/min. Ketoconazole concentrations: (a) 150 μg/g, (b) 100 μg/g. Peaks: 1 = ketoconazole; 2 = clotrimazole (internal standard).

Fig. 3. Separation of ketoconazole from lung (a), plasma (b) and liver (c) of the rat. For chromatographic conditions see Fig. 2b. Ketoconazole concentration: (a) 23.9 μg/g, (b) 28.9 μg/ml, (c) 72.2 μg/g. Peaks: 1 = ketoconazole; 2 = clotrimazole (internal standard).

analysis of ketoconazole in the adrenals since a major endogenous component extracted from the tissue eluted with virtually the same retention time as the drug (Fig. 2). Separation of ketoconazole from the interfering peak in extracts of adrenal glands was achieved by the use of a ternary mobile phase containing methanol, acetonitrile and phosphate buffer (0.02 *M*, pH 6.8). The separation was optimized by varying the ratios of the three components in the mobile phase until a baseline resolution was achieved. The optimal mobile phase was methanol—acetonitrile—0.02 *M* phosphate buffer (pH 6.8) in the ratio 35:30:35 (Fig. 2). Under these conditions the retention times of ketoconazole and clotrimazole were 3.8 min (k' = 7.6) and 6.0 min (k' = 11.0), respectively. The overall analysis time was 7.5 min. The ternary mobile phase described was equally well suited to the analysis of ketoconazole in plasma, liver and lung of the rat (Fig. 3).

The limits of detection (defined as the concentration of drug in the tissue which gave a signal-to-noise ratio of 3:1) were 200 ng/ml in plasma, 400 ng/g in lung and liver and 25 μg/g in adrenal. The relatively high limit of detection for the adrenal was due to the small size of the organs which typically weighed between 10 and 20 mg. However, the ketoconazole levels found in the adrenal were high and the drug could still be detected in these organs 24 h after oral administration of 150 mg/kg ketoconazole.

TABLE I

ABSOLUTE RECOVERIES OF KETOCONAZOLE AND CLOTRIMAZOLE FROM SPIKED LIVER, LUNG, ADRENAL AND PLASMA OF THE RAT

Tissue	Recovery (mean ± S.D.) (%)	
	Ketoconazole	Clotrimazole
Plasma*	85.0 ± 9.5	79.3 ± 9.2
Liver*	91.3 ± 3.8	84.1 ± 8.4
Lung*	93.6 ± 6.5	80.7 ± 11.9
Adrenal**	87.1 ± 9.2	79.6 ± 9.2

*For both drugs, n = 5. Amounts of 50, 40, 30, 20 or 10 μg of ketoconazole were added to 1 ml of plasma or 500 mg of lung and liver; 300 μg of clotrimazole were added to each sample.
**For both drugs, n = 5. Amounts of 5, 4, 3, 2 or 1 μg of ketoconazole were added to a single adrenal gland; 30 μg of clotrimazole were added to each sample.

TABLE II

ACCURACY AND PRECISION DATA FOR THE ASSAY OF KETOCONAZOLE IN LIVER, LUNG, ADRENAL AND PLASMA OF THE RAT

For each tissue, n = 6.

Tissue	Concentration (μg/g or μg/ml)		Relative standard deviation (%)
	Added	Found	
Plasma	50.0	51.4	3.33
	5.0	5.15	3.14
Liver	40.0	42.0	3.71
	—	44.0*	3.20
Lung	40.0	42.5	3.37
Adrenal	200.0	195.0	3.96

*Six determinations made on the same liver from a rat previously treated with ketoconazole.

The absolute recoveries (Table I) of ketoconazole and clotrimazole were determined by comparing their peak heights in a calibration curve with those obtained upon the injection of solutions in 0.01 M hydrochloric acid containing the same original concentration of the two drugs. For ketoconazole, the absolute recoveries ranged between 85.0 (plasma) and 93.6% (lung). The absolute recoveries for clotrimazole ranged between 79.3 (plasma) and 84.1% (liver).

In all cases, the peak-height ratios were linearly related to the amount of drug added to the biological medium ($r > 0.998$), and the between-day reproducibility of the slopes (coefficient of variation, n = 4) was less than 5% for each tissue. The accuracy and precision of the procedures were determined by analysing six samples of each biological medium spiked with ketoconazole (Table II). In addition, six 500-mg sections were taken from the same liver of an animal which had been dosed 2 h previously with ketoconazole. The reproducibility (relative standard deviation) ranged between 3.14%

for plasma (5 μg/ml) and 3.96% for the adrenal (200 μg/g) (Table II). All the concentrations of the drug were found to be within 6% of those added to the tissues. It is interesting to note that the relative standard deviation for the six liver sections spiked with ketoconazole was approximately the same as that found in the six samples taken from the liver of a rat dosed with ketoconazole. This suggests that ketoconazole is evenly distributed within the tissues of the liver. The variability in the distribution of the drug between the adrenals and lungs of rats is discussed in the next section.

Tissue distribution of ketoconazole in the rat following oral administration

To demonstrate the application of the described analytical procedure, the distribution of ketoconazole in the rat following oral administration was studied. Three rats were sampled at each time point. Single samples of plasma, both adrenals, both lungs and two samples of liver were assayed at each time point. Peak concentrations were achieved after 2 h in the liver and adrenal and after 4 h in the plasma and lungs (Table III). The highest concentration of drug was found in the adrenal (195.9 μg/g), followed by the liver (90.3 μg/g), plasma (44.9 μg/ml) and lungs (24.7 μg/g) (Table III). Although preliminary, these data (Table III) indicate that the drug is eliminated from the plasma, liver, adrenal and lung at approximately the same rate.

Considerable variation in the mean levels of the drug found in the tissues of the three animals sampled at each time point was observed (Table III). This is probably due to variation in the rates of absorption and elimination of the drug between animals. However, within a single animal the levels found in both lungs or in both the samples of liver varied by less than 10%. In contrast the levels found in the left and right adrenals from the same animal varied by as much as 150%.

In summary, a sensitive, accurate and reproducible method for the determination of ketoconazole in rat tissues has been developed. The procedure permits the tissue distribution of the drug to be monitored following oral administration for up to 24 h in the adrenal and up to 48 h in the plasma, lung and liver.

TABLE III

DISTRIBUTION OF KETOCONAZOLE IN RAT TISSUES FOLLOWING ORAL ADMINISTRATION OF 150 mg/kg IN CORN OIL SUSPENSION

Time (h)	Concentration (mean ± S.D.) (n = 3)			
	Plasma* (μg/ml)	Liver** (μg/g)	Lung** (μg/g)	Adrenal** (μg/g)
0.5	27.78 ± 4.61	72.30 ± 32.35	18.08 ± 8.58	119.26 ± 45.07
1.0	20.52 ± 6.95	70.97 ± 17.53	20.20 ± 4.08	152.20 ± 36.95
2.0	35.47 ± 3.48	90.28 ± 11.85	24.24 ± 10.02	195.88 ± 67.38
4.0	44.91 ± 15.85	80.39 ± 6.63	24.65 ± 8.64	154.60 ± 76.94
8.0	24.43 ± 5.45	55.08 ± 4.23	16.62 ± 1.96	100.06 ± 19.94
24.0	16.40 ± 4.22	16.22 ± 11.16	4.64 ± 2.92	90.86 ± 46.61
48.0	1.42 ± 1.51	0.71 ± 1.55	0.56 ± 0.42	<25.00

*The mean of a single plasma assay from three rats.

**The mean of duplicate samples from three rats.

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