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Note

## High-performance liquid chromatographic analysis of miconazole in plasma

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Miconazole is a synthetic imidazole derivative with broad-spectrum antifungal activity [1—3]. It is relatively non-toxic and has been used experimentally in the treatment of various systemic mycoses, including candidiasis, coccidiodomycosis, cryptococcosis and histoplasmosis. Interest prevails in evaluating the bioavailability and distribution of various oral formulations of the drug and comparing them with formulations intended for intravenous (i.v.) administration.

Therefore, an analytical method suitable for specifically measuring parent drug levels in plasma was sought. Although gas chromatography with electron-capture detection has been used for monitoring miconazole [4—6], this technique has disadvantages associated with it. One of the reported procedures [4] requires derivatization of the drug prior to chromatography to avoid poor peak shapes (pronounced tailing). The authors suggest that subsequent to derivatization the drug may be pyrolyzed in the injection port and, therefore, only a fragment of the parent is detected. Under these conditions, the ability of the method to differentiate between parent drug and potential metabolites is questionable. A $^{63}$Ni source was used and resulted in limited linearity of detector response. The other method [5,6] employed a Sc$^{3}$H detector source to circumvent the limited detector linearity associated with $^{63}$Ni. However, because of the restricted detector temperature ($\leq$ 300°C) with Sc$^{3}$H, the detector was not self-cleaning for miconazole and after ca. 50 determinations, the Sc$^{3}$H foil had to be removed and cleaned in an ultrasonic bath. These methods reported an overall recovery of 50—75% from biological fluid.

To avoid the problems of (a) thermal decomposition, (b) need for derivatization, (c) limited detector linearity and lifetime, and (d) mediocre recovery of drug, a high-performance liquid chromatographic (HPLC) method for miconazole has been developed with the drug being detected spectrophotometrically.

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## EXPERIMENTAL

### *Apparatus*

Chromatography was performed on a component system consisting of a Waters Assoc. (Milford, MA, U.S.A.) Model 6000A pump, Model U6K injector and Model 440 absorbance detector operated at 254 nm. A Waters μBondapak $C_{18}$ (30 cm × 3.9 mm I.D.; 10 μm particle size) column was used throughout.

### *Materials*

Miconazole nitrate was obtained from Prodotti Chimici Farmaceutici (Rome, Italy) and the internal standard, R-18,648 was obtained from Janssen Pharmaceutica (Beerse, Belgium). Both were used without further purification. Miconazole (free base) was obtained by neutralization of an aqueous solution of the nitrate with sodium hydroxide and extraction into chloroform. Evaporation of the chloroform extract yielded a yellow oil which was recrystallized from benzene—hexane (10:90) to yield crystals of the free base, m.p. 79—81°C (elemental analysis calculated for $C_{18}H_{14}N_2OCl_4$, C=51.9; H=3.4; N=6.7; found C=51.98; H=3.50; N=6.60).

### *Separation of miconazole from dog blood*

Blood samples (3 ml) were centrifuged at 1100 *g* for 10 min. A 1-ml aliquot of the resulting plasma was transferred to a 15-ml glass centrifuge tube, 25 μg of internal standard, R-18, 648 (25 μl of a 1 mg/ml stock solution prepared in methanol) and 1 ml of a 0.2 *M* sodium hydroxide solution (final pH ca. 12.6) was added and the mixture agitated. A 5-ml aliquot of *n*-heptane—isoamyl alcohol (98.5:1.5) was then added and the mixture extracted with vortex mixing. The layers were separated by centrifugation and the plasma layer frozen in a dry ice—acetone bath. The organic layer was then transferred by decantation into a second 15-ml glass centrifuge tube. The plasma was extracted a second time as described above and the two organic layers were combined. The extract was evaporated to dryness under a stream of nitrogen in a 60°C water bath, and the residue dissolved in 100 μl of HPLC grade methanol (Fisher Scientific, Pittsburgh, PA, U.S.A.).

### *Chromatography*

A 60-μl aliquot of the reconstituted extract was chromatographed as an octanesulfonate ion-pair on an octadecylsilane bonded phase column thermostated at 50°C with UV absorbance detection of the effluent at its $\lambda_{max}$, 254 nm. A ternary mobile phase of methanol—tetrahydrofuran—2.5 mmol/l aqueous acetate buffer, pH 5 (62.5:5:32.5) containing 5 mmol/l octanesulfonate was used for separation. Flow-rate was maintained at 2.0 ml/min.

### *Quantitative analysis*

Miconazole was quantitated by determining the analyte:internal standard (R-18,648) peak height ratio and comparing this value with a standard curve constructed after analysis of plasma samples containing known amounts of drug. The calibration curve was generated by analyzing two samples of plasma

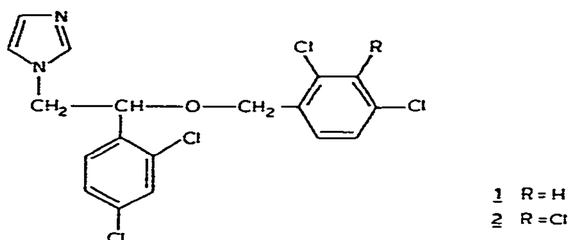
at each of six concentrations of miconazole ranging from 1—50 μg/ml. To determine overall recovery, a second standard curve was constructed by spiking the HPLC mobile phase with miconazole and internal standard at the same concentrations used in the generation of the plasma calibration curve and then subjecting these solutions to direct HPLC analysis.

*Animal studies*

A beagle dog (weighing 11.5 kg) was administered 250 mg of miconazole as an i.v. bolus (administration time, 1 min) via the jugular vein. Blood samples (3 ml) were drawn from the other jugular vein 5, 10, 15, 30, 60, 120, 180, 240, 300 and 360 min post-dosing. Samples were centrifuged and plasma stored at 4°C until analyzed (within 48 h).

## RESULTS AND DISCUSSION

Analysis of miconazole, 1, in plasma is carried out in two stages: initial extraction of the drug from the biological mixture into a water-immiscible solvent and chromatographic separation of the resulting extract on a reversed-phase column with spectrophotometric monitoring of the column eluent.




*Extraction*

Plasma was initially alkalinized to pH 12 with sodium hydroxide to present the amine functions in a totally non-ionized form. Subsequent extraction of the plasma with the moderately polar solvent diethyl ether afforded 20% recovery of drug. Extraction efficiency could be increased to 83 ± 2% by 1:1 volume dilution of the (ether) extractant with hydrocarbon (e.g., *n*-hexane). Taking full advantage of the hydrophobic nature of the free base and extracting the pH-adjusted plasma with *n*-heptane containing 1.5% isoamyl alcohol to avoid emulsification and adsorption of drug onto the glass container surfaces, afforded quantitative recovery (100 ± 2%) of drug. The extract contained minimal contamination by other materials present in the plasma as subsequently determined by HPLC. Extractants containing diethyl ether were much less specific and in addition to being less efficient, gave extracts that subsequently produced chromatograms showing significant contamination.

Depending on the animal species used for the bioavailability studies, either 0.1-ml or 1.0-ml blood samples will be drawn. In this study recovery of miconazole was independent of sample size over the concentration range 1—50 μg of drug per ml of plasma.

Overall recovery from sample-to-sample was determined by the addition of a constant amount of internal standard, R-18,648, 2, directly to the plasma

sample. The internal standard chosen was a halogenated analogue of the drug and also was quantitatively recovered (100 ± 2%) by extraction with heptane (containing 1.5% isoamyl alcohol). In all cases two extractions of plasma with 2.5 volumes of heptane were required for quantitative recovery of 1 and 2.

*Chromatography*

Extracts were separated on an RP-18 column. Initial attempts at separation involved methanol—water or acetonitrile—water mobile phases with and without sodium octanesulfonate as ion-pairing agent. These systems were incapable of separating the drug from the internal standard or from co-extracted contaminants. Peak shape and resolution could be improved by thermostating the column at an elevated temperature (e.g., 50°C). Under these conditions, capacity factor ($k'$) for all components decreased, a situation that could be balanced by decreasing the volume fraction of organic modifier in the mobile phase. With this system optimum resolution was achieved using methanol—2.5 mmol/l aqueous acetate buffer, pH 5 (75:25) containing 1 mmol/l octanesulfonate mobile phase. For most samples, this eluent offered adequate resolution of 1 and 2 from contaminants with short analysis times ($t_R$ = 5.5±0.5 min). Unfortunately, about 20% of the plasma samples contained a contaminant (inter-animal variation) that co-eluted with the internal standard. By reducing the methanol concentration in the mobile phase and concomitantly adding a small amount of tetrahydrofuran (a more powerful organic modifier), a ternary eluent of methanol—tetrahydrofuran—2.5 mmol/l aqueous acetate buffer, pH 5 (62.5:5:32.5) containing 6 mmol/l octanesulfonate was finally selected which was capable of resolving all peaks of interest. The column was still maintained at 50°C to minimize band broadening. Under these conditions miconazole and internal standard eluted with $k'$ values of 3.7 and 5.5, respectively (Fig. 1). Equal sensitivity was gained by monitoring the column effluent spectrophotometrically at 254 or 280 nm; however, at 254 nm contributions to the chromatogram from plasma contaminants were minimized and this wavelength was therefore chosen for subsequent analysis.

*Quantitative analysis*

Miconazole was quantitated by comparing peak height ratio (drug:internal standard) for the unknown with a standard curve prepared by adding known amounts of miconazole and a constant amount of internal standard to plasma samples and carrying the resulting solutions through the analysis sequence. A typical curve generated after analysis of six duplicate plasma samples containing 1—50 μg of 1 and 25 μg of 2 per ml is described by the equation, peak height ratio = 6.99·(concentration of 1) + 8.56 (correlation coefficient 0.998) (Fig. 2). To determine overall recovery of 1 from plasma, a second standard curve was constructed after analysis of samples of 1 prepared in mobile phase (at the same concentration employed with the curve generated from plasma samples) and then immediately analyzed by HPLC (without extraction). The resulting line is described by peak height ratio = 6.96·(concentration of 1) — 3.20 (correlation coefficient = 0.999) (Fig. 2). Overall recovery, determined as the ratio of the slopes of these two calibration plots, was 100%.

The injection-to-injection reproducibility of analysis was determined by

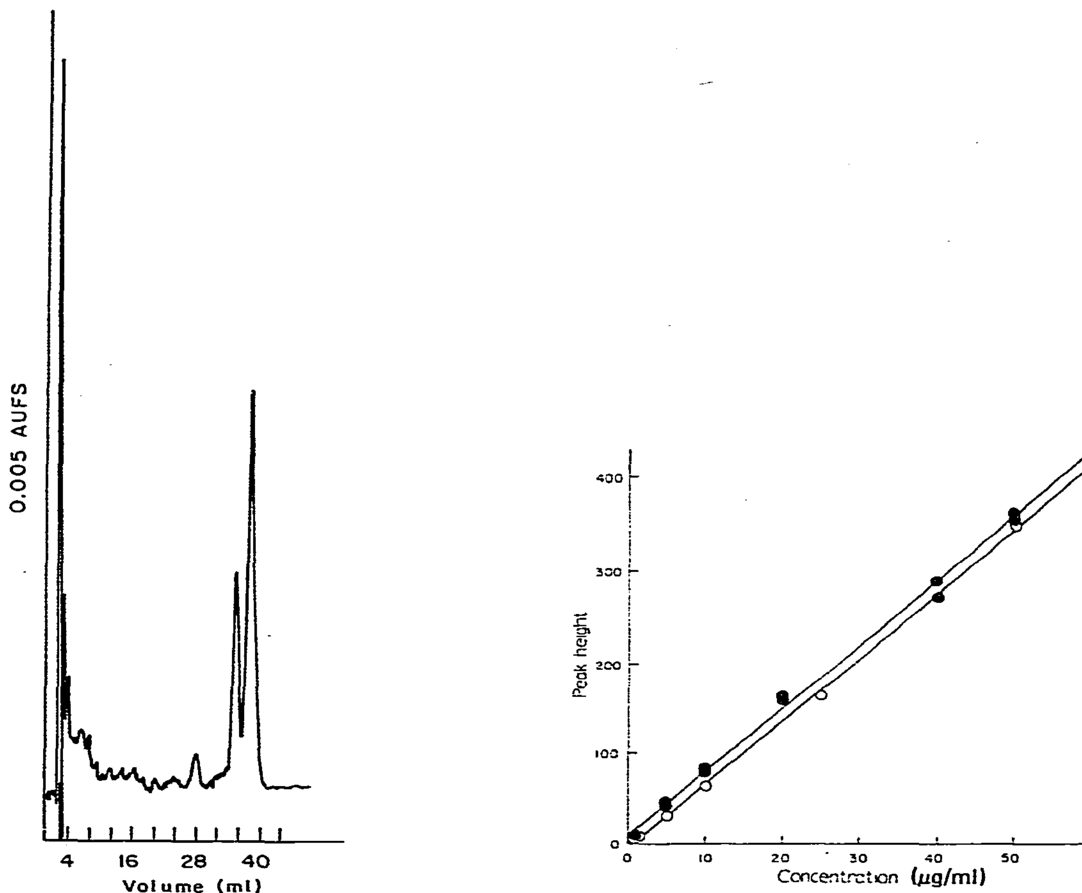



Fig. 1. Chromatogram of miconazole from dog plasma. Miconazole (1 μg/ml of plasma), retention volume, 28 ml; internal standard (25 μg), retention volume, 38 ml. Separation carried out on octadecylsilane bonded phase column with 6 mmol/l sodium octanesulfonate in methanol—tetrahydrofuran—2.5 mmol/l aqueous acetate buffer, pH 5 (62.5:5:32.5) as the mobile phase. Eluent monitored at 254 nm. Flow-rate 2 ml/min.

Fig. 2. Calibration curve for analysis of miconazole from dog plasma (•). Plasma was spiked with known amounts of miconazole and carried through the analysis sequence. Calibration curve generated by HPLC analysis of samples containing known amounts of miconazole prepared directly in mobile phase (○) (without extraction or other pretreatment of samples).

repetitive injection of a single sample containing 10 μg/ml of miconazole. A relative standard deviation of ± 0.4% was obtained from five replicate injections. Sample-to-sample variation was determined by analysis of six plasma samples spiked with 30 μg of miconazole per ml and six containing 1 μg of miconazole per ml, representing high and low concentrations in bioavailability studies. At the high concentration, a relative standard deviation of ± 5% was observed, whereas at the lower level (1 μg/ml), a relative standard deviation of ± 1% was determined. The trend toward lesser variance at lower drug levels was not expected, and cannot be explained at this time. The sensitivity limit for the method determined from analysis of increasingly lower concentrations of drug in plasma was 250 ng/ml at a signal-to-noise ratio of 3:1.

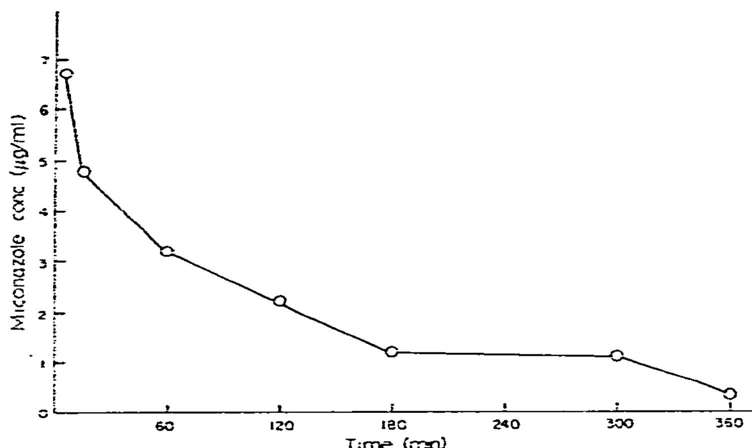



Fig. 3. Plasma levels of miconazole present in a dog administered the drug i.v. (250 mg) over a 1-min period.

*Bioavailability studies*

To demonstrate the applicability of the method for evaluating distribution of various dosage forms of miconazole for i.v. administration, a standard dose (250 mg) of drug was administered to a beagle dog. The plasma—time profile (Fig. 3) demonstrates peak levels of 7 µg/ml declining to levels of ca. 500 ng/ml at 6 h post-dose. Elimination of the drug from plasma can thus be followed for an adequate period of time to evaluate and describe the distribution pattern.

In conclusion, a simple, rapid HPLC method is described for analysis of miconazole in plasma. Although it lacks the sensitivity of methods previously described based on gas—liquid chromatographic separation with electron-capture detection, it is sufficiently sensitive to monitor the drug after i.v. dosing. In addition, it overcomes the difficulties associated with these gas—liquid chromatographic procedures of (a) thermal decomposition of the drug, (b) need for derivatization, (c) limited detector linearity and/or lifetime and (d) poor overall recovery of drug from plasma.

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