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Journal of Chromatography B, 715 (1998) 436–440

JOURNAL OF CHROMATOGRAPHY B

Short communication

Simple high-performance liquid chromatographic method for determination of ketoconazole in human plasma

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Received 16 March 1998; received in revised form 19 May 1998; accepted 19 May 1998

Abstract

A simple high-performance liquid chromatographic method using fluorescence detection was developed for the determination of ketoconazole in human plasma. The method entailed direct injection of the plasma sample after deproteinization using acetonitrile. The mobile phase comprised 0.05 *M* disodium hydrogen orthophosphate and acetonitrile (50:50, v/v) adjusted to pH 6. Analysis was run at a flow-rate of 1.5 ml/min with the detector operating at an excitation wavelength of 260 nm and an emission wavelength of 375 nm. The method is specific and sensitive with a quantification limit of approximately 60 ng/ml and a detection limit of 40 ng/ml at a signal-to-noise ratio of 3:1. Mean absolute recovery value was about 105%, while the within-day and between-day coefficient of variation and percent error values of the assay method were all less than 14%. The calibration curve was linear over a concentration range of 62.5–8000 ng/ml.

Keywords: Ketoconazole

1. Introduction

Ketoconazole is an imidazole type of oral broad-spectrum antifungal agent used in the treatment of superficial and systemic fungal infections [1–3]. Various analytical methods including microbiological assay [4–7] and high-performance liquid chromatography (HPLC) [8–13] have been developed for the determination of ketoconazole in biological samples. The microbiological assays based on the method of agar well diffusion may not be sufficiently specific for measurement of the drug in plasma, since these methods detect total antifungal activity of the plasma but not that of the drug alone. HPLC methods using ultraviolet (UV) detection were reported by Alton [8], Andrews et al. [9], and Mannisto et al. [11], but all these methods required elaborate sample preparation. Alton [8] and Mannisto et al. [11] reported the use of a multiple liquid–liquid extraction procedure while Andrews et al. [9] used a solid-phase extraction procedure. Both extraction methods appeared to be tedious and time consuming. Moreover, no internal standard was employed for quantification of ketoconazole in the methods of Alton [8] and Andrews et al. [9]. Also, the method of Andrews et al. [9] was not validated for accuracy, precision, and sensitivity. Furthermore, the column required to be heated during analysis. HPLC method using fluorescence detection was reported recently by Swezey et al. [10]. This method however required extensive washing of extraction solvent prior to sample preparation. In addition, a method using electrochemical

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0378-4347/98/$19.00
PII: S0378-4347(98)00253-9

detection has also been reported by Hoffman et al [13] with high sensitivity. However, the electrochemical detector may require frequent cleaning or polishing of the working electrode to restore the sensitivity. Hence, it may not be so suitable to be used for bioavailability studies, which involve a large number of samples to be analyzed.

In this paper, we report a specific and sensitive HPLC method which requires simple sample preparation, for the determination of ketoconazole in human plasma using fluorescence detection. We also demonstrated the applicability of this method in a bioavailability study.

2. Experimental

2.1. Materials

Acetonitrile and methanol, HPLC grade were purchased from Mallinckrodt (Kentucky, USA). Disodium hydrogen orthophosphate anhydrous was purchased from Fisher Scientific (New Jersey, USA). Ketoconazole standard was obtained from United State Pharmacopeia (MD, USA).

2.2. Instrumentation

The HPLC system comprised a Jasco PU-980 pump, a Jasco 821-FP spectrofluorometer detector (Jasco, Tokyo, Japan), a Rheodyne 7125 sample injection valve fitted with a 50 μl sample loop (Rheodyne, California, USA), and a Hitachi D-2500 Chromato-integrator (Hitachi, Tokyo, Japan). The detector was operated using a gain of 100, an attenuation of 32, an excitation wavelength of 260 nm and an emission wavelength of 375 nm. A Metaphase KR100-5-C18 (Bioscience, Kuala Lumpur, Malaysia) column (5 μm, 250×4.6 mm I.D.) fitted with a refillable guard column (Upchurch Scientific, Oak Harbour, WA, USA) packed with Perisorb RP-18 (30–40 μm, pellicular), was used for the chromatographic separation. The mobile phase comprised 0.05 *M* disodium hydrogen orthophosphate anhydrous and acetonitrile (50:50, v/v) adjusted to pH 6 with glacial acetic acid. Analysis was run at a flow-rate of 1.5 ml/min and the samples were quantified using peak height.

2.3. Sample preparation

A 250 μl aliquot of plasma sample was measured into an Eppendorf microcentrifuge tube and deproteinized by adding 250 μl of acetonitrile. The mixture was vortexed for 1 min using a vortex mixer and then centrifuged at 12 800 g for 30 min. The supernatant was transferred into a new eppendorf microcentrifuge tube and 50 μl injected onto the column.

2.4. Assay validation

Standard calibration curves were constructed by spiking drug-free pooled plasma with a known amount of ketoconazole at a concentration range of 62.5–8000 ng/ml. These plasma standards were also used to determine the absolute recovery, within-day and between-day precision and accuracy ($n=6$) of the method. The absolute recovery ($n=6$) was estimated by comparison with directly injected drug solution of corresponding concentrations. The ketoconazole stock solution was prepared by dissolving 100 mg of ketoconazole in 100 ml of methanol to achieve a concentration of 1000 μg/ml. The working ketoconazole standard solutions were then prepared by serial dilutions of the ketoconazole methanolic stock solution with distilled water. The drug solution was stable for at least two months at room temperature.

3. Results and discussion

The method employed in our study involved direct injection of the plasma sample after precipitation with a deproteinization agent. During assay development, different deproteinization agents which included perchloric acid, trichloroacetic acid, tribromoacetic acid, methanol, and acetonitrile were employed in the sample preparation. It was found that the recovery of ketoconazole was less than 20% when perchloric acid, trichloroacetic acid, and tribromoacetic acid were used for the deproteinization of plasma samples. This finding suggests that the presence of acidic deproteinization agents may lead to a loss of the drug, presumably due to the drug being bound strongly to the plasma proteins. Hence,

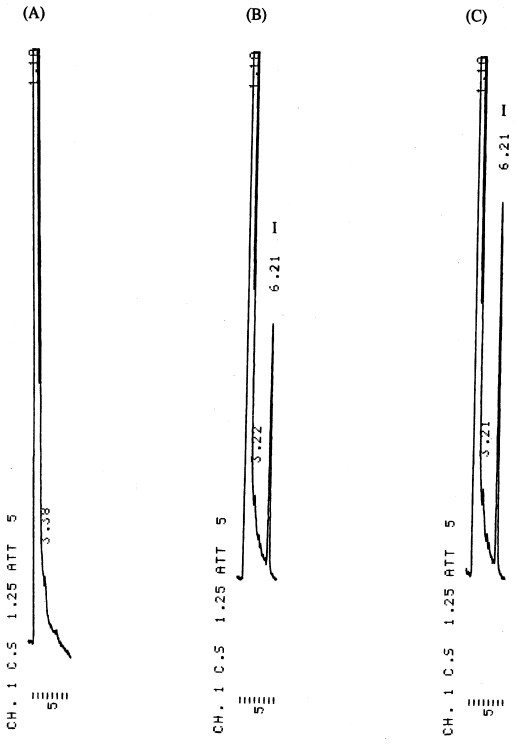

Fig. 1. Chromatograms for the analysis of ketoconazole in plasma. (A) Blank plasma. (B) Plasma spiked with 2000.0 ng/ml ketoconazole. (C) A volunteer plasma containing 3258.0 ng/ml ketoconazole 3 h after oral administration of 200 mg of ketoconazole. (*Y*-axis: Attenuation=5; *X*-axis: Chart speed=2.5 mm/min; I=ketoconazole.)

precipitation of the plasma proteins resulted in a loss of the ketoconazole in the supernatant. Such a loss in drug concentration was not observed when the acidic deproteinization agents were added into aqueous solutions of the drug. On the other hand, there was no appreciable loss of ketoconazole when methanol and acetonitrile were used for the deproteinization, with recovery values of approximately 100%. However, methanol produced a more turbid sample and hence more dirty chromatogram compared to that of acetonitrile. Based on these findings, acetonitrile was chosen as the deproteinization agent in the present study. However, it was found that an equal volume of acetonitrile to plasma was required to obtain a reasonably clean sample. When the amount of acetonitrile was reduced, the sample obtained was turbid indicating that deproteinization may not be sufficiently complete. Although an equal volume of acetonitrile was required for the deproteinization, with subsequent dilution of the plasma sample, the method remained very sensitive, achieving a detection limit of 40 μg/ml at a signal-to-noise ratio of 3:1, being more sensitive than the methods of Alton [8], Andrews et al. [9], Swezey et al. [10], and Badcock [12]. Moreover, an internal standard was not required in our study as a linear curve was obtained between the peak height and ketoconazole concentration in the plasma over the range of concentrations (62.5–8000.0 ng/ml) measured.

Chromatograms obtained with blank plasma and plasma spiked with ketoconazole are shown in Fig. 1A Fig. 1B, while that of a volunteer 3 h after dosing with 200 mg ketoconazole is shown in Fig. 1C. It can be seen that the ketoconazole peak with a retention time of 6.2 min, was well resolved and free of interference from endogenous compounds in the plasma. In addition, the total run time for each sample was only 8 min.

Table 1
Absolute recovery, within-day and between-day precision and accuracy (n=6)

Concentration (ng/ml)	Recovery		Between-day		Within-day	
	Mean (%)	C.V.%	Accuracy (%)	Precision (C.V.%)	Accuracy (%)	Precision (C.V.%)
62.5	113.7	4.9	112.0	12.5	113.6	5.3
125.0	100.9	4.3	90.5	11.3	112.8	4.6
250.0	103.4	5.6	99.7	11.9	98.7	4.0
500.0	116.4	3.9	99.1	7.2	93.9	3.9
1000.0	100.4	2.5	106.7	7.0	95.1	4.5
2000.0	99.9	2.2	97.2	3.5	96.3	2.3
4000.0	102.2	1.1	99.5	2.1	100.9	1.2
8000.0	101.4	2.0	98.8	1.6	101.1	2.0

The mobile phase used in the present study consisted only of phosphate buffer and acetonitrile. In the methods of Andrews et al. [9] and Badcock [12], a third component, namely, diethylamine, was added into the mobile phase. It was also found that the pH of the mobile phase was found to be critical in separating the drug from endogenous compounds, achieving optimum resolution at a pH of 6. At pH value of less than 6, the elution time of the ketoconazole peak was correspondingly reduced and the peak was not well separated from an adjacent interfering peak gradually overlapping as the pH value was reduced to 4.

The absolute recovery, within-day and between-day accuracy and precision values are presented in Table 1. The average absolute recovery value was about 105% with a coefficient variation of 3.3%, while the coefficient of variation (C.V.) and percent error values of the within-day and between-day precision and accuracy were all less than 14%. The

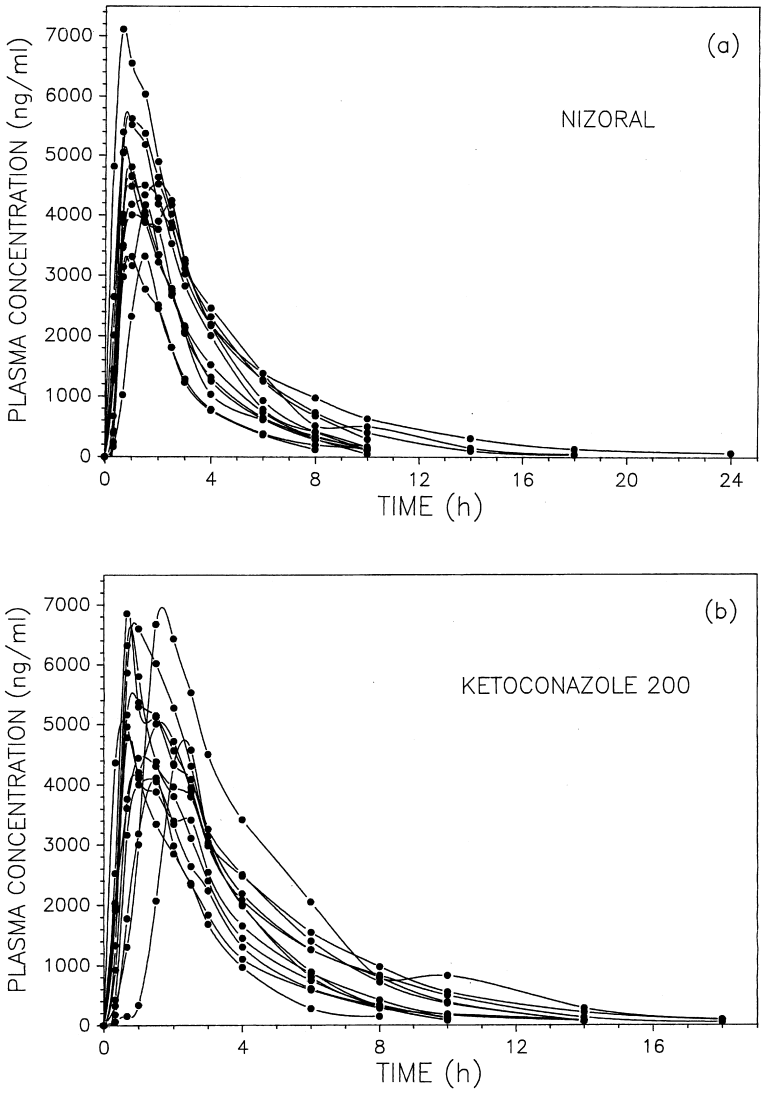

Fig. 2. Plasma ketoconazole concentration versus time profiles from 12 volunteers following oral administration of 200 mg of Nizoral and Ketoconazole 200.

standard calibration curve ($n=6$) was found to be linear over the concentration range used with a correlation coefficient of 0.9999. The drug in the plasma was found to be stable for at least 2 months when stored at −30°C. A quantification limit was set at 60 ng/ml being not more than 5% lower than the lowest concentration used to construct the standard curve.

The present method was applied to analyze plasma samples of 12 healthy adult male volunteers from a comparative bioavailability study of two different ketoconazole tablet preparations, namely, Nizoral and Ketoconazole 200, the latter being a generic preparation. Fig. 2 shows the individual plasma concentration–time profiles of the volunteers obtained with the two preparations. It can be seen from the plasma profiles of both preparations that for majority of volunteers ketoconazole could still be detected up to 10 h, and in some up to 18 and 24 h. In all cases, the last detectable level was less than 5% of the peak plasma concentration.

In conclusion, the present HPLC method was simple, specific, sensitive, and suitable to be used for determination of ketoconazole in pharmacokinetic/bioavailability studies.

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