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Short communication

Rapid method for the analysis of itraconazole and hydroxyitraconazole in serum by high-performance liquid chromatography

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Abstract

An assay for the determination of itraconazole and its active metabolite hydroxyitraconazole in serum has been developed, using ketoconazole as internal standard. The procedure involved a one-step liquid–liquid extraction of the drug, its metabolite and the internal standard, followed by their separation by reversed-phase HPLC. In this paper the validation of this method is described.

Keywords: Itraconazole; Hydroxyitraconazole

1. Introduction

Itraconazole is a triazole antifungal drug with a broad spectrum of activity, which is administered orally. Its absorption is maximal if taken after meals. Maximum serum levels appear approximately 3–4 h after ingestion and its terminal half-life is about 20 h. Itraconazole is extensively metabolized in the liver and over 30 metabolites have been found. Of the metabolites only hydroxyitraconazole is active. Each metabolite represents <1–5% of the parent compound, except for hydroxyitraconazole, that reaches serum concentrations 2–3 times higher than itraconazole [1]. A total 'active level' (itraconazole plus hydroxyitraconazole) of 1 mg/l is considered as optimal for clinical efficacy in fungal infections [2]. Because of a wide inter-individual variability in absorption, serum concentration monitoring is warranted in patients having severe fungal infections. Several methods are described for the analysis of itraconazole, but without the active metabolite hydroxyitraconazole [3,4]. Only one method is known by us which also describes the analysis of hydroxyitraconazole [5]. This method, however, uses a back-extraction which is time-consuming. Therefore we developed a new method which is quicker than the published method with the same analytical recovery. In this paper we describe the validation of this method according to Dutch standards [6].

2. Experimental

2.1. Instrumentation and chromatographic conditions

All chromatographic analysis were performed on a solvent delivery system Model 300 (Separations, H.I.

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Ambacht, Netherlands), a 717^{plus} autosampler (Waters, Etten-Leur, Netherlands) and a variable-wavelength detector Polychrom 9065 (Varian, Houten, Netherlands). Integrations and calculations were carried out on a Varian LC Star-system and a Highscreen kompakt serie^{III} computer.

The HPLC column was a Lichrospher 10RP8, 250×4.6 mm I.D. (Chrompack, Middelburg, Netherlands) and the flow-rate of the mobile phase was 2.0 ml/min. The analysis took place at ambient temperature at a wavelength of 258 nm.

2.2. Reagents

Itraconazole, hydroxyitraconazole and ketoconazole were kindly supplied by Janssen (Beerse, Belgium). Heptane, isoamylalkohol, diethylamine, acetic acid and sodium carbonate (all of analytical grade) were purchased from Merck (Amsterdam, Netherlands).

Acetonitrile (HPLC-grade) was obtained from Rathburn Brunschwig (Amsterdam, Netherlands), methanol from Baker (Deventer, Netherlands), sodium carbonate and sodium hydrogencarbonate from OPG (Utrecht, Netherlands).

Carbonate buffer pH 10 was prepared by adding 53.4 ml of 1 *M* sodium carbonate to 46.6 ml of 1 *M* sodium hydrogen carbonate.

2.3. Mobile phase and extraction solvent

The mobile phase consisted of acetonitrile–water (62:38, v/v), to which 0.05% diethylamine was added. The pH was adjusted to 6.0 with 30% acetic acid and the mobile phase was degassed ultrasonically for 10 min before use.

The extraction solvent was heptane–isoamylalcohol (90:10, v/v).

2.4. Standards

Itraconazole and hydroxyitraconazole were obtained from Janssen as methanolic solutions of 20 μg/ml each. Ketoconazole was used as internal standard at a concentration of 40 μg/ml methanol. All standard solutions were stored at −20°C.

2.5. Sample collection and preparation

Serum was collected by centrifuging blood tubes at 2500 *g* for 5 min. The serum was transferred into a polypropylene test tube and stored at −20°C. Standards were made by spiking newborn bovine serum with itraconazole and hydroxyitraconazole. Calibration curves were made over the range 0.1–1.6 mg/l.

To 1 ml of serum (standards, quality control and unknown) 1.0 μg of ketoconazole (internal standard) was added, then 200 μl of 1 *M* carbonate buffer (pH 10) was added and the samples were vortex-mixed for 3 s. For the extraction 7 ml heptane–isoamylalcohol (90:10, v/v) was added and the mixture was vortex-mixed for 1.5 min. After centrifugation (5 min, 2200 *g*) the organic layer was separated and evaporated to dryness at 37°C by air. The residue was dissolved in 125 μl of mobile phase and 40 μl was injected into the HPLC system.

The detection wavelength was 258 nm and peak-height ratios were used for calculations.

3. Results

Fig. 1 shows two examples of chromatograms, one obtained from blank bovine serum (A) and one obtained from patient serum spiked with internal standard (B). No interferences from commonly coadministered drugs such as antimicrobials, oncolytics and analgesics were observed.

3.1. Extraction efficiency

The recoveries of itraconazole and hydroxyitraconazole were 77±5% and 83±3% respectively; both estimated over the range 0.1–3.2 mg/l (n=6). The recovery of ketoconazole was 79±3% (n=6).

3.2. Precision

The day-to-day precision was estimated over three different concentrations (0.1, 0.7 and 2.0 mg/l). The samples were stored at −20°C and measured over five working days. The results are presented in Table 1.

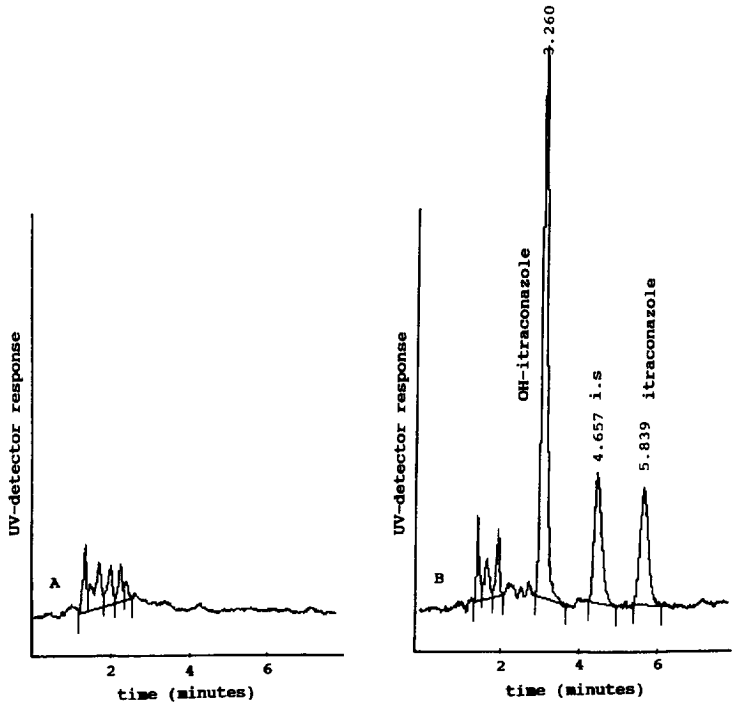

Fig. 1. Chromatograms of (A) blank bovine serum and (B) human serum; the concentrations of itraconazole and hydroxyitraconazole correspond to 0.34 and 1.01 mg/l respectively.

3.3. Linearity

The linearity was tested and demonstrated over the range 0.1–3.2 mg/l with the goodness-of-fit test.

Fig. 2 and Fig. 3 show calibration curves.

3.4. Limit of detection

At a signal-to-noise ratio of 3 the lower limit of detection was 10 μg/l for itraconazole and 7 μg/l for hydroxyitraconazole.

Table 1
Day-to-day variation of itraconazole and hydroxyitraconazole.

Compound	Concentration (mg/l)	n	Coefficient of variation (%)
Itraconazole	0.1	5	4.2
Hydroxyitraconazole	0.1	5	6.0
Itraconazole	0.7	5	3.7
Hydroxyitraconazole	0.7	5	3.4
Itraconazole	2.0	5	7.9
Hydroxyitraconazole	2.0	5	5.6

3.5. Stability of the samples

Serum samples from patients receiving itraconazole were demonstrated to be stable during six months storage at −20°C. In addition, the methanolic standard solutions of itraconazole and hydroxy-

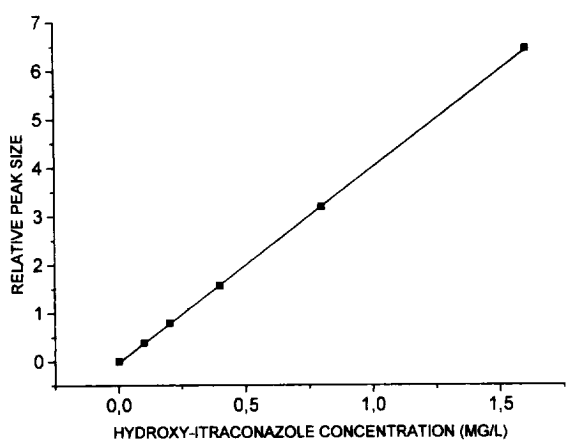

Fig. 2. Calibration curve of itraconazole; $R^2=0.9999$

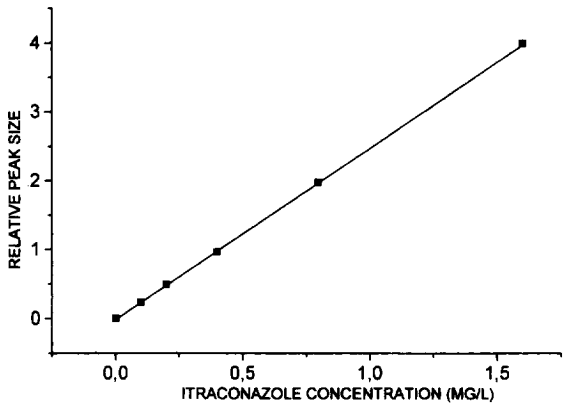

Fig. 3. Calibration curve of hydroxyitraconazole; R^2=0.9999.

itraconazole, stored at 4°C, were stable for at least twelve months.

4. Discussion

With our method we were able to determine both itraconazole and hydroxyitraconazole. This is an advantage over the methods described by Woestenborghs et al. [3] and Salim et al. [4], because hydroxyitraconazole is an important metabolite [7]. Poirier et al. [5] have also described an assay for both itraconazole and hydroxyitraconazole. Compared with their method, our extraction procedure is much quicker while the results are similar. Poirier et al. used a back-extraction for an extra cleaning of their samples. We found that when an extraction with heptane–isoamylalcohol (90:10, v/v) was performed, no back-extraction was needed to get the same results.

Our method was sensitive enough to measure the samples of patients who were included in our study (hematologic patients). They took two itraconazole tablets of 100 mg per day.

Until now we have analysed serum samples of more than one hundred patients and the analytical results are very satisfactory.

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