A sensitive and selective high-performance liquid chromatographic method was developed for the determination of itraconazole and its active metabolite, hydroxyitraconazole, in human plasma. Prior to analysis, both compounds together with the internal standard were extracted from alkalinized plasma samples using a 3:2 (v/v) mixture of 2,2,4-trimethylpentane and dichloromethane. The mobile phase comprised 0.02 M potassium dihydrogen phosphate-acetonitrile (1:1, v/v) adjusted to pH 3.0. Analysis was run at flow-rate of 0.9 ml/min with excitation and emission wavelengths set at 260 and 365 nm, respectively. Itraconazole was found to adsorb on glass or plastic tubes, but could be circumvented by prior treating the tubes using 10% dichlorodimethylsilane in toluene. Moreover, rinsing the injector port with acetonitrile helped to overcome any carry-over effect. This problem was not encountered with hydroxyitraconazole. The method was sensitive with limit of quantification of 3 ng/ml for itraconazole and 6 ng/ml for hydroxyitraconazole. The calibration curve was linear over a concentration range of 2.8–720 ng/ml for itraconazole and 5.6–720 ng/ml for the hydroxy metabolite. Mean recovery value of the extraction procedure for both compounds was about 85%, while the within-day and between-day coefficient of variation and percent error values of the assay method were all less than 15%. Hence, the method is suitable for use in pharmacokinetic and bioavailability studies of itraconazole.

**Keywords:** Itraconazole; Hydroxyitraconazole

1. Introduction

Itraconazole (Fig. 1A) is an orally active triazole antifungal agent, which is active against a broad spectrum of fungal species including Cryptococcus, Candida, Aspergillus, Blastomyces and Histoplasma capsulatum var capsulatum [1,2]. Its oral bioavailability was found to increase when taken with food, with plasma concentrations approximately two-times that taken in the fasted state [3,4]. It is extensively metabolized in the liver and the metabolite hydroxyitraconazole (Fig. 1B), has been identified to be active [5].

Various microbiological and high-performance liquid chromatography (HPLC) methods have been reported for the determination of itraconazole in biological fluids following oral administration. However, the microbiological procedures are not only less sensitive but also lack specificity, since itraconazole has an active metabolite which contributes to the antifungal activity [6–8].

On the other hand, some of the HPLC methods are tedious and time consuming, involving multiple extraction steps in the sample pre-treatment [6,9,10]. Moreover, the methods by Woestenborghs et al. [9] and Warnock et al. [6] only quantified the parent drug but not the hydroxy metabolite. A method using solid phase extraction was reported by Rifai et al. [11], but again it was only for the quantification of parent drug. In addition, the accuracy of the method was not reported and the limit of quantification was set at 50 ng/ml even though the linearity was reported to range from 250 to 1000 ng/ml. Other methods reported by Ng et al. [12], Darouiche et al. [13] and Allenmark et al. [14] were also for quantifying the parent drug and not the active metabolite. Meanwhile, Compas et al. [15], Gubbins et al. [16] and Al-Rawithi [17] described methods that could quantify both the parent drug and hydroxy metabolite. However, all
these methods involved protein precipitation in the sample pre-treatment. While allowing rapid sample clean-up, these methods possessed relatively low sensitivity with limit of quantification ranging from 25 to 100 ng/ml, which might not be suitable for pharmacokinetic or bioavailability studies where lower concentrations need to be quantified. Another method that also involved direct deproteinization was reported by Cox et al. [18]. This method however, used a big injection volume of 190 μl to compensate for the loss in sensitivity during the protein deproteinization process. Moreover, the mobile phase utilized a low pH of 2.45 which might lead to rapid deterioration of the column used. Also, the method had a relatively long run time of approximately 21 min.

Methods based on liquid-chromatography–mass spectrometer (LC/MS) have also been reported for the quantification of itraconazole in plasma samples [19,20]. However, the applicability of these methods is limited as the equipment is not widely available.

In this paper, we report a sensitive and specific HPLC method for the determination of both itraconazole and hydroxyitraconazole in human plasma using fluorescence detection with a run time of less than 12 min. The assay method was evaluated for accuracy, precision, recovery and linearity for both compounds. The method has a limit of quantification of 2.8 and 5.6 ng/ml for itraconazole and hydroxyitraconazole, respectively, being suitable for use in pharmacokinetic/bioavailability studies. The sample preparation involved a one-step liquid–liquid extraction followed by washing with hexane to produce cleaner samples for prolonging the column life. The applicability of the method was demonstrated by applying it to analyze plasma samples obtained from a bioavailability study.
2. Experimental

2.1. Materials

Potassium dihydrogen phosphate, potassium carbonate, glacial acetic acid and hydrochloric acid were purchased from Merck (Darmstadt, Germany). Itraconazole, hydroxyitraconazole and the internal standard (R051012) (Fig. 1C) were kindly supplied by Janssen Research Foundation (Beerse, Belgium). All other solvents used were of spectrophotometric, AR or HPLC grade, purchased from Mallinckrodt (Kentucky, USA).

2.2. Instrumentation

The HPLC system comprised a Jasco PU-980 pump (Jasco, Tokyo, Japan), a FP-1520 fluorescence detector (Jasco, Tokyo, Japan), a Rheodyne 7125 sample injector (Rheodyne, California, USA) and a Hitachi D-2500 Chromato-integrator (Hitachi, Tokyo, Japan). A Genesis CN (Jones Chromatography Ltd., UK) column (4 μm, 250 mm × 4.6 mm i.d., 120 Å) fitted with a refillable guard column (Upchurch Scientific, WA, USA) packed with Perisorb RP-18 (30–40 μm, pellicular) was used for the chromatographic separation. The mobile phase consisted of acetonitrile and 0.02 M potassium dihydrogen phosphate (50:50, v/v) and the mixture was adjusted to pH 3.0 with 5 M hydrochloric acid and delivered at a flow-rate of 0.9 ml/min. The whole chromatographic system was operated at room temperature (25 °C) with an excitation wavelength of 260 nm and emission wavelength of 365 nm. The gain was set at 100 × while the attenuation was set at 32.

2.3. Sample preparation

A 500 μl volume of plasma sample was accurately measured into a 10 ml glass tube with a PTFE-lined screw cap, followed by the addition of 100 μl of 0.45 μg/ml internal standard methanolic solution, 100 μl of 1 M potassium carbonate solution and 5 ml of premixed extracting solvent (2,2,4-trimethylpentane: dichloromethane, 7:3, v/v). The mixture was then vortexed for 2 min on a vortex mixer and centrifuged at 3500 rpm for 10 min. The organic layer was transferred into a reactivial and evaporated to dryness at 84 °C under a gentle stream of nitrogen gas. The residue was reconstituted with 50 μl of a mixture solution consisting of 1 M acetic acid and acetonitrile (3:2, v/v), followed by the addition of 500 μl of hexane. The mixture was vortexed for 1 min and centrifuged at 2500 rpm for 5 min. The hexane layer was then discarded and 50 μl of the aqueous layer was then injected onto the column. Samples were quantified using peak height ratio of itraconazole or hydroxyitraconazole and internal standard peaks were well resolved and free of interference from endogenous compounds in the plasma, with retention times of 9.7, 6.7 and 10.8 min, respectively. The total run time for each sample was less than 12 min.

During assay development, several solvents namely, dichloromethane, chloroform, diethyl ether, heptane and 2,2,4-trimethylpentane at different ratios were used to extract the three compounds from the plasma samples. A 7:3 mixture of 2,2,4-trimethylpentane and dichloromethane was found to provide the cleanest chromatogram and also highest recovery values during a single extraction for all three compounds. Mean recovery values of 93.2, 85.4 and 79.5% were obtained for hydroxyitraconazole, itraconazole and the internal standard, respectively. Increasing the 2,2,4-trimethylpentane to a ratio of 8:2 led to lower recovery (less than 70%) of hydroxyitraconazole. On the other hand, when the dichloromethane content was increased, recovery of the internal standard was markedly reduced (less than 65%).

3. Results and discussion

Chromatograms obtained with blank plasma and plasma spiked with 11.3 ng/ml itraconazole, 11.3 ng/ml of hydroxyitraconazole and 0.45 μg/ml of internal standard are shown in Fig. 2A and B. It can be seen that the itraconazole, hydroxyitraconazole and internal standard peaks were well resolved and free of interference from endogenous compounds in the plasma, with retention times of 9.7, 6.7 and 10.8 min, respectively. The total run time for each sample was less than 12 min.

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The type and molarity of the alkalization agent used to adjust the pH of the plasma prior to the extraction, was found to be critical in achieving cleaner chromatograms with fewer
interfering endogenous peaks. When sodium hydroxide was used as the alkalization agent instead of potassium carbonate, there were additional endogenous compounds interfering with the hydroxyitraconazole and itraconazole peaks. A similar observation was seen when saturated potassium carbonate solution was employed.

The extraction recovery, within-day and between-day accuracy and precision values for itraconazole are presented in Table 1 while those for hydroxyitraconazole in Table 2. For itraconazole, the coefficient of variation (CV, %) values of both the within-day and between-day precision were all less than 12%, while those of accuracy had percent error values of less than 9%. In the case of hydroxy metabolite, the respective precision and percent error values were all less than 15%. The standard calibration curve ($n = 6$) for itraconazole was found to be linear over the concentration range of 2.8–720 ng/ml with a slope of −0.02 and an intercept of 0.03 ($X$: concentration, $Y$: ratio of the peak height of drug to internal standard). The linear range for hydroxyitraconazole ($n = 6$) was 5.6–720 ng/ml and the values for the slope and intercept obtained were 0.03 and 0.15, respectively. Both compounds have correlation coefficient of 0.999. The limit of quantification was set at 3 and 6 ng/ml for itraconazole and hydroxyitraconazole, respectively, being the lowest concentrations used in the respective calibration curves. These values are comparable to those using LC/MS methods reported by Yao et al. [19] and Carrier and Parent [20]. For itraconazole, the limit of quantification of
Table 1
Extraction recovery, within-day and between-day precision and accuracy (n = 6) for itraconazole

<table>
<thead>
<tr>
<th>Concentration (ng/ml)</th>
<th>Extraction recovery</th>
<th>Within-day</th>
<th>Between-day</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean (%) CV (%)</td>
<td>Precision (CV, %) Accuracy (error, %)</td>
<td>Precision (CV, %) Accuracy (error, %)</td>
</tr>
<tr>
<td>2.8</td>
<td>89.3 9.1</td>
<td>11.6</td>
<td>3.9 4.3</td>
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<td>76.9 6.1</td>
<td>8.9</td>
<td>0.1 5.1</td>
</tr>
<tr>
<td>11.3</td>
<td>86.4 9.2</td>
<td>3.2</td>
<td>2.6 9.1</td>
</tr>
<tr>
<td>22.5</td>
<td>89.3 9.8</td>
<td>7.4</td>
<td>−8.7 9.6</td>
</tr>
<tr>
<td>45</td>
<td>89.4 6.3</td>
<td>3.1</td>
<td>8.8 10.6</td>
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<tr>
<td>90</td>
<td>77.0 8.5</td>
<td>0.9</td>
<td>8.9 7.3</td>
</tr>
<tr>
<td>180</td>
<td>86.1 7.3</td>
<td>7.9</td>
<td>0.6 4.1</td>
</tr>
<tr>
<td>360</td>
<td>89.1 8.7</td>
<td>4.4</td>
<td>1.2 5.3</td>
</tr>
<tr>
<td>720</td>
<td>85.4 8.1</td>
<td>5.9</td>
<td>−4.4 6.9</td>
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</table>

Table 2
Extraction recovery, within-day and between-day precision and accuracy (n = 6) for hydroxyitraconazole

<table>
<thead>
<tr>
<th>Concentration (ng/ml)</th>
<th>Extraction recovery</th>
<th>Within-day</th>
<th>Between-day</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean (%) CV (%)</td>
<td>Precision (CV, %) Accuracy (error, %)</td>
<td>Precision (CV, %) Accuracy (error, %)</td>
</tr>
<tr>
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<td>94.6 6.6</td>
<td>10.7</td>
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<td>92.2 3.8</td>
<td>14.3</td>
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<tr>
<td>720</td>
<td>94.3 5.2</td>
<td>5.1</td>
<td>−4.3 1.7</td>
</tr>
</tbody>
</table>

The present method is lower than that reported by Cox et al. [18] and Poirier et al. [10] where the reported values were 10 and 20 ng/ml, respectively.

The itraconazole and hydroxyitraconazole in plasma samples were found to be stable for at least 3 months when stored frozen in −20°C. In addition, the methanolic standard solutions for both drugs were also found to be stable for at least 6 months when stored at 4°C.

Although adsorption of itraconazole to glassware and plastic tube has not been previously reported, this phenomenon was observed during assay development resulting in poor linearity of serially diluted calibration curves. This was confirmed by extracting glass and plastic tubes pre-exposed to itraconazole with the extraction solvent. A drug peak was observed when the extracts were analyzed using the HPLC method, but not with those from glass or plastic not previously exposed to the drug. The problem was circumvented by coating the glasswares with 10% dichlorodimethylsilane in toluene. Also, rinsing the injector port with acetonitrile helped to overcome any carry-over effect. However, the problem was not encountered with hydroxyitraconazole.

The present method was applied to analyze plasma samples of 12 healthy adult male volunteers obtained from a bioavailability study of an itraconazole capsule preparation. Fig. 2C shows a chromatogram obtained from the plasma sample of a volunteer taken 4.0 h after a 100 mg dose of itraconazole, while Fig. 3 shows the plasma concentration-time profiles of itraconazole and its metabolite, hydroxyitraconazole obtained from a volunteer who participated in the study. In all 12 volunteers, the smallest quantifiable itraconazole and hydroxyitraconazole concentrations in the pharmacokinetic study were less than one-seventh of the respective peak plasma concentrations. This would enable the pharmacokinetic parameters such as the elimination rate constant (k_e) and total area under the plasma concentration-time curve (AUC_0→∞) to be estimated with a reasonably level of accuracy.

Fig. 3. Plasma concentration vs. time profiles from one volunteer following oral administration of 100 mg of itraconazole (■) hydroxyitraconazole, (○) itraconazole.
In conclusion, the present HPLC method was sensitive, specific and suitable to be used for determination of plasma itraconazole and hydroxyitraconazole in pharmacokinetic/bioavailability studies.

References
