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ORIGINAL RESEARCH

A rapid HPLC method with fluorometric detection for determination of plasma itraconazole and hydroxy-itraconazole concentrations in cystic fibrosis children with allergic bronchopulmonary aspergillosis

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ABSTRACT: The development and validation of a simple, rapid and selective high-performance liquid chromatography (HPLC) method is described for the quantitation of itraconazole and hydroxy-itraconazole in 100 μL of plasma from a paediatric population. The mobile phase of methanol (75% v/v) and water (25% v/v) was pumped at 1 mL/min through a C_{18} *Symmetry*™ (3.9 mm i.d. × 150 mm) cartridge. Using a protein-precipitation method, 100 μL internal standard (IS) solution (R051012, 555 μg/L in acetonitrile) were added to 100 μL of plasma followed by 10 μL zinc sulphate solution (20% w/v). Itraconazole, hydroxy-itraconazole and IS eluted at 4.7, 8.3 and 12.5 min, respectively and were detected fluorometrically at 250 nm (excitation) and 380 nm (emission). Recoveries were 87.1–96.7%. Calibrations in drug-free plasma were linear ($r^2 > 0.99$) from 50 to 2000 μg/L, using $1/c^2$ (c = concentration) weighting. Intraday and interday imprecision (CV%) was 4.8–17.3 and 6.3–16.6% for itraconazole, and 4.6–17.9 and 7.02–18.4% for hydroxy-itraconazole. Inaccuracy was −7.1 to −14.7% for itraconazole and −0.1 to −9.7% for hydroxy-itraconazole. The clinical application of this method was demonstrated by measurement of itraconazole and hydroxy-itraconazole in plasma samples drawn from paediatric cystic fibrosis patients, who were prescribed itraconazole for treatment of allergic bronchopulmonary aspergillosis. Copyright © 2005 John Wiley & Sons, Ltd.

KEYWORDS: Itraconazole; hydroxy-itraconazole; high–performance liquid chromatography (HPLC); cystic fibrosis

INTRODUCTION

Itraconazole (*Sporonax*®) is used widely in the treatment and prophylaxis of a variety of fungal infections, especially *Aspergillus* species, which can cause a hypersensitivity and inflammatory reaction, called allergic bronchopulmonary aspergillosis (ABPA) that can result in damage to lung tissues (Van Cutsem, 1989). It is given mainly by the oral route (as capsules), but also as an aqueous solution with a cyclodextrin as a solubilizer (Stevens, 1999). In some countries it is also available as an injectable solution (Janssen Pharmaceutica Products, 2002). The pharmacokinetics of orally administered itraconazole are of continuing interest, partly because of its very poor water solubility, which contributes to low, variable absorption, but also because it is both an inhibitor and substrate of the cytochrome P450 3A4 enzyme and the P-glycoprotein transporter system and therefore causes interactions with several other drugs (Angirasa and Koch, 2002; Sachs *et al.*, 1993; Wang *et al.*, 2002). It is metabolized by the cytochrome P450 system (Florea *et al.*, 2003; Kramer *et al.*, 1990; Varis *et al.*, 2000) to over 30 metabolites. One of these metabolites, hydroxy-itraconazole, has a similar antifungal spectrum to the parent and is therefore considered the main metabolite. (De Beule *et al.*, 2001; Van Cutsem, 1989). These factors collectively introduce considerable variability into the pharmacokinetics and, therefore, the clinical response. Some centres now routinely request plasma concentration monitoring of itraconazole alone or with hydroxy-itraconazole in some circumstances (Conway *et al.*, 2004; Cox *et al.*, 1997; Summers *et al.*, 1997). There are a number of existing HPLC assays (Badock, 1990; Brandsteterova *et al.*, 1995; Compas *et al.*, 1996; Gubbins *et al.*, 1998; Koks *et al.*, 2002; Ohkubo and Osanai, 2005; Poirier *et al.*, 1994; Poirier and Cheymol, 1997; Remmel *et al.*, 1988; Rifai *et al.*, 1995; Srivatsan *et al.*, 2004; Woestenborghs *et al.*, 1987; Wong *et al.*, 2003) for such applications which are more selective, sensitive, rapid and precise than microbiological methods (Hostetler *et al.*, 1993). Assays using HPLC with mass spectrometric detection (Vogeser *et al.*, 2003; Yao *et al.*, 2001) have advantages in terms of sensitivity, selectivity and the need for extensive sample workup, but not all laboratories have the

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Abbreviations used: ABPA, allergic bronchopulmonary aspergillosis; CF, cystic fibrosis.

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resources to support this technology. Furthermore, the turnaround time may be unacceptable if requests have to be batched with LC-MS/MS systems on which higher priorities are placed for other drugs.

A method was required, which was rapid, robust, accurate, sensitive and precise and which could cope with the small sample volumes drawn from very young CF children who had ABPA. We took advantage of the fluorescent properties of the triazole functional groups of itraconazole to obtain sufficient selectivity and sensitivity when a simple one-step protein precipitation step was used for the sample cleanup. The development, performance and clinical application of such an assay are described herein.

EXPERIMENTAL

Chemicals and reagents. Itraconazole (R051211), hydroxy-itraconazole (R06373) and R051012 (Fig. 1) were supplied by Janssen-Cilag Pty Ltd, North Ryde, NSW, Australia. HPLC-grade acetonitrile and methanol were obtained from Merk KGaA, Darmstadt, Germany. Zinc sulfate was obtained from Ajax Chemicals Pty Ltd, Auburn, NSW, Australia. Drug-free plasma was supplied by the pathology laboratory of the Mater Children's Hospital, Mater Health Services, South Brisbane, Qld, Australia. Deionized water (HPLC-grade, 18 MΩ), was supplied by the Production Unit, Mater Pharmacy Services, Mater Children's Hospital, South Brisbane and was used throughout.

HPLC instrumentation and chromatographic conditions. A Shimadzu (Kyoto, Japan) model LC-10AS pump and Shimadzu model RF-551 fluorometric detector were connected to a model 712 WISP autosampler (Millipore, Billerica, MA, USA), a column oven (Millipore), and strip chart recorder (Kipp & Zonen, Delft, Netherlands). The mobile phase of methanol (75% v/v) and water (25% v/v) was prepared daily and filtered and degassed through a 0.45 μm pore-size membrane (Millipore) under negative pressure. The column was a C_{18} *Symmetry*™ (3.9 mm i.d. × 150 mm; Waters, Milford, MA, USA) maintained at 30°C. The flow rate was 1 mL/min and the detector was set to excitation and emission wavelengths of 250 and 380 nm, respectively.

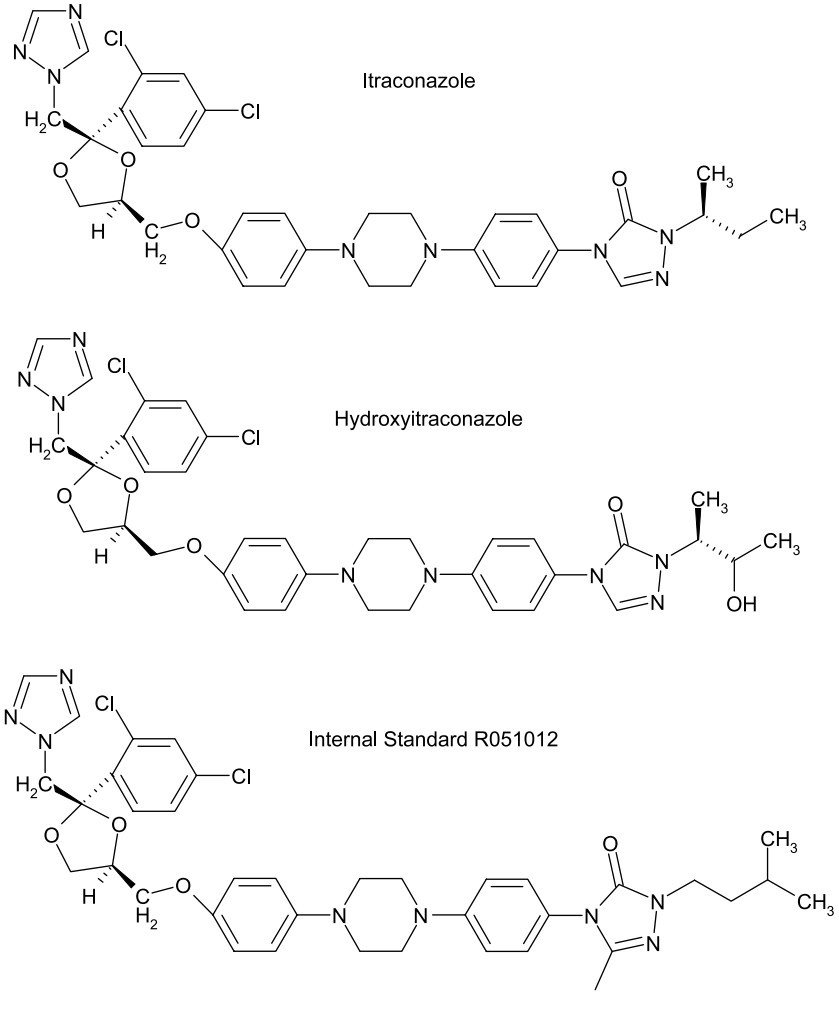

Figure 1. Structure of itraconazole, hydroxy-itraconazole and R051012 (internal standard).

Table 1. Imprecision, accuracy and recovery of itraconazole and hydroxy-itraconazole

Concentration (μg/L)	Recovery (%) (n = 4, ±SD)	Inaccuracy (%) (n = 6)	Imprecision (CV%) Intraday (n = 3)[a]	Imprecision (CV%) Inter-day (n = 6)
Itraconazole				
75	96.6 ± 6.8	–9.2	17.3	16.6
200	87.1 ± 2.8	–14.7	8.0	6.3
1750	88.3 ± 7.0	–7.1	4.8	7.8
Hydroxy-itraconazole				
75	95.8 ± 23.7	–9.7	17.9	18.4
200	87.9 ± 2.3	–7.0	7.9	12.2
1750	96.7 ± 8.0	–0.1	4.6	7.0

SD, standard deviation; CV, coefficient of variation.
[a] Intraday CV was calculated using three batches of QC concentrations on two consecutive days.

Sample preparation. Plasma (100 μL) was pipetted into labelled Eppendorf 2 mL tapered polypropylene tubes. Ten microlitres of a zinc sulfate solution (20% w/v) and 100 μL of R051012 solution in acetonitrile (555 μg/L) were added to each tube, which were vortex-mixed for 10 s and then centrifuged at ~9300 ***g*** (relative centrifugal force) for 5 min. A 50 μL portion of the clear supernatant was injected.

Inaccuracy, imprecision, recovery, sensitivity. Inaccuracy (%Acc) was calculated as the percentage of the back-calculated average values (from six independent sets of calibration data; $c_{ave,obs}$) of the seeded controls to their respective nominal values (c_{nom}; Table 1):

$$\%\,\mathrm{Acc} = [(c_{ave,obs} - c_{nom})/c_{nom}] \times 100$$

Imprecision was estimated as the percentage coefficient of variation (%CV) over the concentration range of low, middle and high seeded controls (Table 1):

$$\%\,\mathrm{CV} = [\text{standard deviation (SD)}/c_{ave,obs}] \times 100$$

Absolute recoveries were calculated by comparison of the peak heights of processed samples (H_{plasma}) with those from the direct injection of the appropriate amount of analyte in mobile phase ($H_{mobile\ phase}$):

$$\%\ \text{absolute recovery} = (H_{plasma}/H_{mobile\ phase}) \times 100$$

Mean recoveries were obtained from four determinations (n = 4) of each of the three seeded controls for both itraconazole and hydroxy-itraconazole (Table 1).

Calibration standards and seeded controls. Master stock solutions of itraconazole and hydroxy-itraconazole (250 mg/L) were prepared in methanol from which working stock solutions (2.5 mg/L) were prepared and used to supplement drug-free human plasma to obtain a series of seven standards from 50 to 2000 μg/L. The plasma standards were stored in 200 μL portions at −80°C until used. Likewise, an independent series of three seeded controls containing both itraconazole and hydroxy-itraconazole (75, 200 and 1750 μg/L) was prepared and stored at −80°C. Itraconazole and hydroxy-itraconazole methanolic solutions have been reported to be stable at 4°C for at least 12 months and serum samples were stable for >6 months at –20°C (Compas *et al.*, 1996; Srivatsan *et al.*, 2004).

Clinical application. Blood samples (n = 98; median = four samples per subject) were drawn by finger prick from 23 children (aged 1–14 years) with CF who were being treated for *Aspergillus* colonization in their lungs. These patients were taking itraconazole as prescribed by their doctor, either as capsules or oral solution. The itraconazole and hydroxy-itraconazole plasma concentrations in plasma were measured as described above.

RESULTS AND DISCUSSION

This method's appeal lies largely in its simplicity while maintaining sufficient precision, accuracy and sensitivity to measure itraconazole and hydroxy-itraconazole in plasma samples of young children receiving treatment for APBA. There is one reported method which used straightforward protein precipitation as the cleanup step, but which required a several-fold larger volume of serum (Gubbins *et al.*, 1998). Chan *et al.* (2003) employed UV detection and their assay was a least 2-fold less sensitive than presently reported. Other methods (e.g. Al-Rawithi *et al.*, 2001) used protein-precipitation in the sample cleanup but employed the common antifungal agent, ketoconazole, as the internal standard. Furthermore, even though triethylamine was included in their mobile phase, the chromatograms still showed peak asymmetry and there was apparent interference with the internal standard peak. Because the water–methanol mobile phase contained no buffer salts or any other additive, it could be left in the column when the system was shut down, thereby permitting more rapid equilibration, which obviated the need for intermediate solvent flushing. Although itraconazole, hydroxy-itraconazole and R051012 each have basic

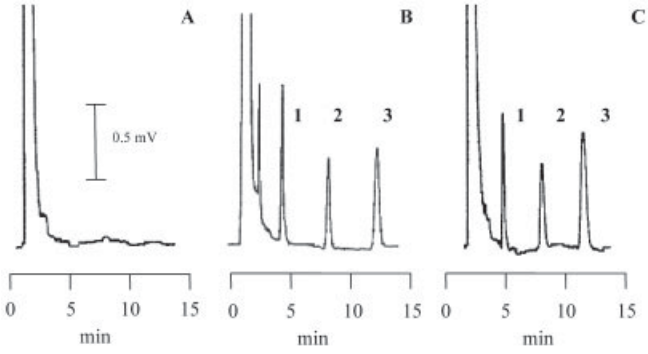

Figure 2. Chromatograms from the assay of a drug-free human plasma (A). Plasma standard containing itraconazole (500 μg/L), hydroxy-itraconazole (500 μg/L) and internal standard (555 μg/L) (B). Plasma sample from a CF patient containing itraconazole (430 μg/L) and hydroxy-itraconazole (419 μg/L) taken 4 h 23 min after a 200 mg dose of itraconazole oral solution (C). Peak identity: **1**, hydroxy-itraconazole (retention time, RT = 4.7 min); **2**, itraconazole (RT = 8.3 min); **3**, R051012 (RT = 12.5 min). Detector response in mV is indicated by the scale bar.

nitrogen-containing functionalities, the use of a 'base-stable' *Symmetry*™ column avoided the need to add triethylamine to improve the peak shapes by 'capping' exposed silanol groups on the column packing. The column has a working life of >1500 injections.

Itraconazole, hydroxy-itraconazole and the IS were eluted at 4.7, 8.3 and 12.5 min, respectively. The chromatograms showed well-resolved peaks with no interference from endogenous substances (Fig. 2). No carryover was observed following injection of mobile phase following the highest (2000 μg/L) standard. Fluorometric detection assisted the straightforward but non-selective protein-precipitation cleanup in maintaining adequate specificity of the chromatography. Analysis of plasma from CF children who were also prescribed fat-soluble vitamins, inhaled and oral steroids, antibiotics (tobramycin, ceftazidime, ticarcillin/clavulanic acid) and various proton-pump inhibitors showed no interference. At least 20 unknown clinical samples can be assayed during a normal working day, and the stability in the autosampler compartment means that samples can be processed unattended overnight.

The goodness of fit (r^2) of the linear regression of peak height ratio on concentration (c) of standards was always greater than 0.99. However, it was deemed necessary to use a $1/c^2$ weighting scheme to ensure satisfactory accuracy at the lower concentrations because of variance-associated distortion of the least-squares fitting procedure from the higher end of the 400-fold concentration range of calibrators. While weighted calibration algorithms may be incorporated into data analysis software supplied with HPLC instrumentation, essentially the same result can be obtained easily on a hand-held calculator by linear regression of appropriately transformed x and y values, as described previously (Wonnacott and Wonnacott, 1970). The lower limit of quantification (LLQ) was set to 75 μg/L. The lower limit of detection (LLD) for peak height of approximately three times average baseline noise was ~40 μg/L. The acceptance criteria during an assay run were set *a priori* according to international standards for biomedical method validation (Shah *et al.*, 2000).

The highest itraconazole and hydroxy-itraconazole concentrations (2067 and 3764 μg/L, respectively) were measured 8 h after the last dose in the same blood sample of a 13-year-old male on 200 mg itraconazole oral solution twice a day (8.8 mg/kg/day). The lowest concentration of itraconazole (75 μg/L) was measured 7.5 h after the last dose in a 10-year-old female who was taking 200 mg itraconazole capsules daily (7.9 mg/kg/day) and the lowest hydroxy-itraconazole concentration (78 μg/L) was measured 1 h after the last dose in a blood sample from a 7-year-old male, who was taking 200 mg itraconazole capsule daily (6.8 mg/kg/day). Measured plasma concentrations (>75 μg/L; n = 82 for itraconazole, n = 69 for hydroxy-itraconazole) are shown in Fig. 3. It has been reported that plasma concentrations, even at steady state, are not predictable from the initial dosing (Summers *et al.*, 1997), and that there is a high intrapatient variability in CF patients receiving itraconazole for treatment of ABPA (Conway *et al.*, 2004). A frequently used strategy is to adjust the dose such that itraconazole concentrations by HPLC exceed 250 μg/L at all times (Summers *et al.*, 1997). For patients in whom we have applied the assay, a change in dose was initiated in two in five cases because plasma samples had itraconazole plasma concentrations less than 250 μg/L.

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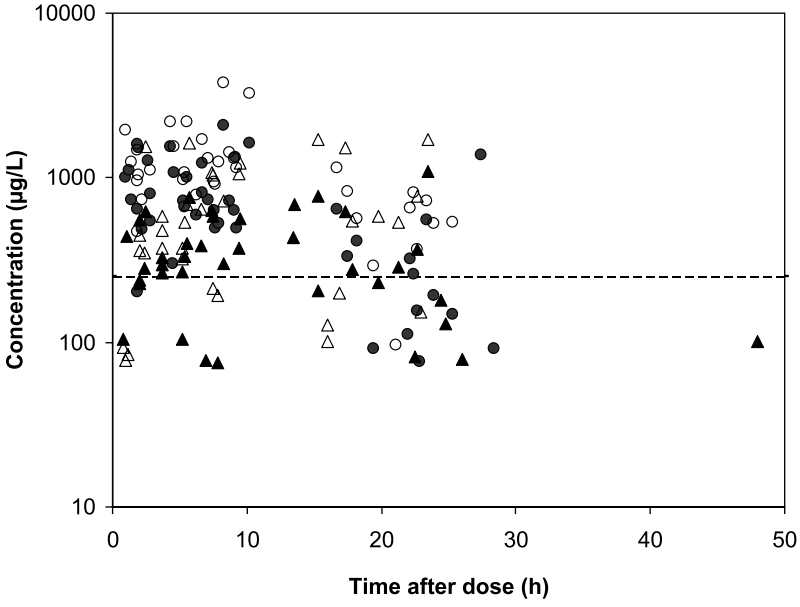

Figure 3. Measured itraconazole and hydroxy-itraconazole plasma concentration (µg/L) in CF children with ABPA. The average dose taken by these patients (aged 8.5 ± 3.3 years) was 6.4 ± 2.2 mg/kg/day. (●) Itraconazole concentrations after taking the oral solution; (○) Hydroxy-itraconazole concentrations after taking the oral solution; (▲) Itraconazole concentrations after taking the capsule; (△) Hydroxy-itraconazole concentrations after taking the capsules. The broken line indicates the recommended minimum therapeutic plasma concentration of itraconazole (Summers *et al.*, 1997).

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