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Note

Micro-scale method for itraconazole in plasma by reversed-phase high-performance liquid chromatography

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For most therapeutic indications, monitoring plasma itraconazole seems unnecessary [1]; however, there are clinical situations, for example in patients with impaired absorption or in patients who fail to respond to the drug, where regular monitoring is important in optimizing therapy. Whilst several microbiological procedures have been developed, they are imprecise [2], have levels of detection inadequate for therapeutic monitoring [2–7] and are not specific since they determine total antifungal activity which might also include a contribution from active metabolites and other antifungal agents; one report has also suggested that only unbound drug may be measured by this method [3]. Recently developed high-performance liquid chromatographic (HPLC) methods [2, 8–10] largely overcome these inadequacies, but require 0.5–2 ml of plasma, which is a decided disadvantage in cases of severe sample limitation, for example in young children on whom frequent other tests may also be required. All but one method, that of Babhair [8], have time-consuming multiple liquid–liquid [2, 9] or column extractions [10] which contribute to their relatively poor recoveries and further suffer loss of analyte during the mandatory evaporation to dryness of their final extract. Babhair [8] attempts to overcome the problem by employing a simple salting-out procedure; this method, however, suffers the disadvantage of using ketoconazole, another broad-spectrum antimycotic agent, as the internal standard and of having a detection limit of 354 nmol/l which is quite inadequate for therapeutic monitoring [2].

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With the current emphasis on economy and the need for rapid, accurate and inexpensive methods adaptable to automation, and in response to requests for antimycotic levels in neonates and children, a cost-effective, quantitative micro-scale assay for itraconazole has been developed which, in using small Reactivials and by incorporating protein precipitation of only 100 μl plasma, overcomes the problems outlined above. This method has been used to investigate the effect of heat inactivation of human immunodeficiency virus (HIV) on itraconazole stability.

EXPERIMENTAL

Reagents and glassware

Acetonitrile (ultraviolet cut-off 190 nm) and methanol are HPLC grade while diethylamine is analytical reagent grade (Ajax Chemicals, Sydney, Australia).

Itraconazole (R51211), (±)-*cis*-4-[4-[4-[4-[[2-(2,4-dichlorophenyl)-2-(1*H*-1,2,4-triazol-1-ylmethyl)-1,3-dioxolan-4-yl]methoxy]phenyl]-1-piperazinyl]phenyl]-2,4-dihydro-2-(1-methylpropyl)-3*H*-1,2,4-triazol-3-one, and the internal standard (R51012), *cis*-4-[4-[4-[4-[[2-(2,4-dichlorophenyl)-2-(1*H*-1,2,4-triazol-1-ylmethyl)-1,3-dioxolan-4-yl]methoxy]phenyl]-1-piperazinyl]phenyl]-2,4-dihydro-5-methyl-2-(3-methylbutyl)-3*H*-1,2,4-triazol-3-one, were obtained as reference compounds from Janssen (Beerse, Belgium).

The stock itraconazole solution, 100 μmol/l (70.6 mg/l) in methanol, is stable for at least six months at 4°C. The working itraconazole solution, 1 μmol/l in methanol, is prepared freshly on the day of analysis.

To prepare the quality control (QC) serum (drug-free pooled human serum containing added itraconazole), rapidly stir 100 ml of serum and slowly add about 3.5 mg of itraconazole; continue stirring the mixture for 1 h. Dilute 50 μl to 10 ml with serum and store in 150-μl aliquots at −20°C; this is stable for at least seven months [11].

The stock itraconazole internal standard solution, 100 μmol/l (73.4 mg/l) in methanol, is stable for at least six months at 4°C. The working itraconazole internal standard solution, 0.25 μmol/l in methanol, is prepared freshly on each day of analysis.

Glass vials, 1.0 ml (Pierce Reactivials, Pierce, Rockford, IL, U.S.A.), were obtained complete with screw caps and PTFE-faced discs.

Sample preparation

To a 1.0-ml Reactivial add 100 μl of plasma or serum. At the same time prepare reagent blank, control (QC) and standard vials. In the standard vials place 5, 10, 50, 100 and 200 μl of itraconazole working solution and evaporate to dryness at 40°C in a stream of dry nitrogen. To the blank and standard add 100 μl of drug-free pooled human serum, to the control add 100 μl of QC serum.

To each vial add 300 μl of working internal standard solution, cap securely and vortex-mix for 60 s; centrifuge at 2000 *g* for 2 min.

High-performance liquid chromatography

Liquid chromatographic analysis is performed using a Model 320 isocratic liquid chromatograph equipped with a Model 165 variable-wavelength detector (Beckman Instruments, San Ramon, CA, U.S.A.). The analytical column is a prepacked 150 mm × 4.6 mm I.D. Ultrasphere ODS, average particle size 5 μm, and the guard column is 50 mm × 4.6 mm I.D. dry-packed with Ultrasphere ODS 20 μm (Beckman Instruments). Chromatography is performed at a constant room temperature of 24 °C.

The samples are eluted isocratically with a water–acetonitrile–diethylamine (40:60:0.05, v/v) mixture at a constant flow-rate of 1.5 ml/min. The solution is prepared daily using double-distilled water, filtered (0.45 μm) and degassed before use. With detector sensitivity 0.02–0.005 a.u.f.s., peak heights at 261 nm are recorded with a 10-mV recorder at a chart speed of 0.25 cm/min.

Inject 250 μl of the supernatant into the chromatograph and elute with the mobile phase. Under the above conditions, the retention time for itraconazole is 6.1 min and for the internal standard 8.5 min. The ratio of peak heights of itraconazole standard to internal standard is calculated and the value for QC and unknown specimens is calculated by direct proportion.

RESULTS AND DISCUSSION

Selectivity, detection limit and linearity

Interference from other antimicrobial agents and drugs likely to be encountered as co-medication was studied. Ketoconazole, miconazole, erythromycin, ampicillin, benzylpenicillin, gentamycin, amoxycillin, bactrim, griseofulvin, cimetidine, zidovidine, prednisolone and diazepam were dissolved in water or methanol at concentrations above their upper therapeutic limits. HPLC determinations were performed as described; none of the above were found to interfere with the analysis. Extracts of plasma samples from patients on amphotericin B, griseofulvin, miconazole, ketoconazole and nystatin also showed no interfering peaks.

Fig. 1 shows a chromatogram of blank plasma and a chromatogram of plasma from a patient being treated with itraconazole. The detection limit, defined as a signal twice the height of the noise level, was approximately 8 nmol/l. Concentration and peak height were linearly related throughout the concentration range investigated, 0.01–6 μmol/l. This adequately covers the range of clinically significant concentrations of the drug in human adult plasma [12].

Analytical recovery and precision studies

Batches of pooled plasma from subjects who were being treated with itraconazole were used for this study. Concentrations of itraconazole were determined using the above procedure. Amounts of itraconazole equivalent to the minimum and maximum concentration investigated, i.e. 0.01 and 6 μmol/l,

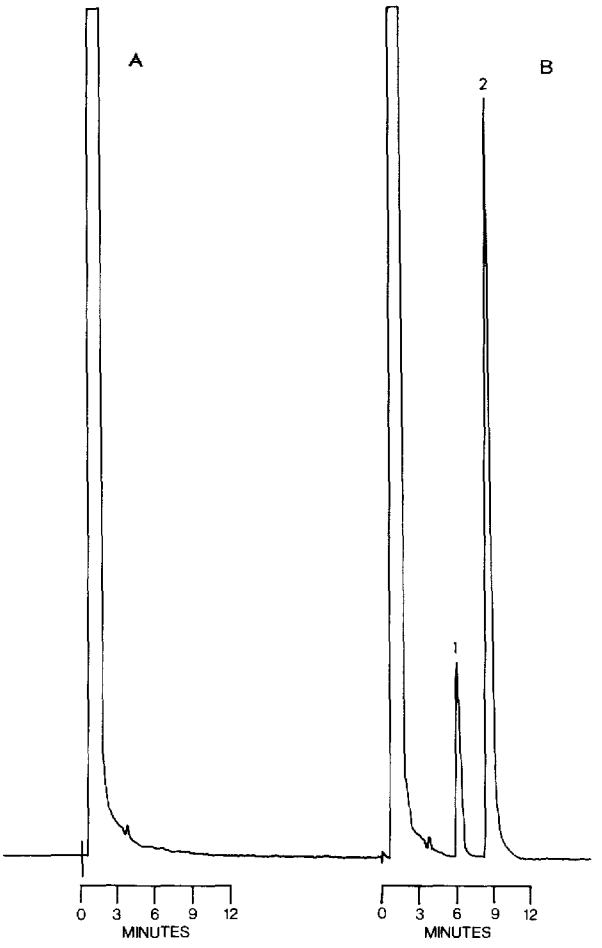

Fig. 1. Chromatograms of (A) extract of drug-free plasma without internal standard and (B) extract of patient plasma sample containing 89 nmol/l itraconazole. Peaks: 1 = itraconazole; 2 = internal standard (R51012).

were added to isotonic saline and to pooled plasma. The preparations were then analysed as above. Absolute recoveries ranged from 95 to 101%. Over a period of four months, the between-run coefficient of variation of samples ($n=9$) having a concentration of 250 nmol/l was 6.2%. Within-run precision was determined by analysis of ten extractions of drug-free plasma that had been supplemented to a concentration of 100 nmol/l; the coefficient of variation was 3.8%.

Effect of heat inactivation of HIV on itraconazole

An increasing number of invasive fungal infections have been reported on patients with acquired immunodeficiency syndrome. As itraconazole is effec-

tive as a fungicidal in immunocompromised hosts [13], it can be reasonably anticipated that a large number of requests for routine itraconazole drug levels will be made on serum-positive (for HIV) subjects. It is well established that heating sera at 56°C for 1 h inactivates HIV [14]; the effect of heat on itraconazole concentration was unknown. This effect was investigated by measuring, in duplicate, itraconazole levels in ten patient plasma samples before and after heating at 56°C for 1 h; with a regression curve $y = 0.989x - 2.073$ and a correlation coefficient of 0.992 between pre- and post-treated samples (x and y nmol/l, respectively), it is quite apparent that such treatment has no effect on itraconazole concentrations.

Chromatographic aspects

Plasma protein is commonly precipitated with organic solvents such as acetonitrile, ethanol or methanol or by simple denaturation with trichloroacetic acid before liquid chromatographic analysis. During development of this assay, it was discovered that when acetonitrile, ethanol, the mobile phase or trichloroacetic acid were used to precipitate the protein and the supernatant was injected, the itraconazole peak was considerably distorted and broad. The decrease in peak height resulted in significantly reduced sensitivity. Substitution of methanol as the deproteinizing agent overcame completely the problem of peak shape as well as considerably simplifying and speeding-up the assay; at a 3:1 (v/v) ratio of methanol to plasma, the supernatant obtained was clear with no micro-precipitate. The analogous step in other HPLC itraconazole methods involving evaporation of the solvent extract to dryness and dissolution in the mobile phase is thus avoided.

The column eluate was monitored at 261 nm, the optimum detection wavelength for itraconazole in the mobile phase used. When monitored at either 280 or 254 nm, the two wavelengths commonly available in fixed-wavelength detectors, reductions of 56 and 8%, respectively, in sensitivity resulted. Similar to that previously noted with a ketoconazole assay [15], fluorescence detection (excitation 245 nm, emission 380 nm) could be used to improve the sensitivity of the assay two-fold, i.e. from 8 to 4 nmol/l; overcoming the background fluorescence of the mobile phase would enable the sensitivity to be even further increased.

After each day's run, the column is washed with methanol–dimethylsulphoxide (90:10, v/v) prior to overnight storage in methanol. This simple procedure has maintained column performance for more than 1000 injections; the practice has always been adopted and so no comment on column life without the daily wash is possible. By employing a conventional 4.6 mm I.D. column, the rapidly increasing back-pressure and column contamination reported by Remmel et al. [10] from the use of a small-bore column (2 mm I.D.) in their itraconazole assay is avoided.

Analysis of plasma samples

Eleven plasma samples from patients taking itraconazole were analysed in duplicate using the above method and the recently described solvent extraction HPLC method of Warnock et al. [2]; levels obtained ranged from 39 to 702 nmol/l. Correlation based on a least-squares linear regression formula gave a line of best fit with a slope of 1.022 and a y-intercept on the solvent extraction method axis of 12.573 nmol/l. The correlation coefficient was 0.989, the results from either assay would appear to be interchangeable.

In conclusion, the procedure described, with its simplicity, rapidity and small volume of plasma required, offers advantages over other HPLC methods. The assay is equally suitable for the routine clinical monitoring and pharmacokinetic study of itraconazole in plasma.

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