Journal of Chromatography, 306 (1984) 436—440
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 1982

Note

Micro-determination of ketoconazole in plasma or serum by high-performance liquid chromatography

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(Received September 14th, 1983)

The relevance of plasma ketoconazole (*cis*-1-acetyl-4-[4-{[2-(2,4-dichlorophenyl)-2-(1H-imidazol-1-ylmethyl)-1,3-dioxolan-4-yl]methoxy}phenyl]-piperazine) levels to clinical response needs further investigation; at present it is probably worth estimating levels only in patients who fail to respond to the drug [1, 2]. To further define this relationship between plasma concentration and therapeutic response in both adults and children, routine monitoring of ketoconazole serum concentration during treatment is necessary, and an accurate, rapid and specific assay method is essential to such studies. Whilst several microbiological procedures have been developed [3—6] they are not specific since they determine the total antifungal activity which might also include a contribution from the active deacyl metabolite [7, 8].

The use of high-performance liquid chromatography (HPLC) for ketoconazole assay has been reported in several recent publications [9—11]. In all of these procedures the sample volume requirement is relatively large (0.5—1 ml), which is a decided disadvantage in cases of severe sample limitation, for example in neonates and small children on whom frequent other tests may also be required. With multiple-step extraction [9] or column elution [10] contributing to relatively poor recoveries but necessary to decrease the amounts of interfering endogenous compounds in plasma, these assays are also time-consuming; in addition, without employment of an internal standard [9, 10] or examination of possible interference from metabolites [10, 11], they may lack accuracy.

In response to requests for drug levels in neonates treated with ketoconazole suspension for systemic mycoses, we have developed a rapid, selective and sensitive assay which involves liquid chromatography and is now in routine use

0378-4347/84/$03.00

for both therapeutic monitoring and pharmacokinetic studies. In contrast with other HPLC methods, for the method we describe as little as 20 μl of plasma or serum are required, sample preparation is simple with the one-step extraction of ketoconazole conducted quickly and conveniently in small vials, and analytical recovery is complete.

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). Ketoconazole, the three postulated metabolites of ketoconazole [9], *cis*-1-acetyl-4-(4-hydroxyphenyl)piperazine (I), *cis*-1-acetyl-4-[4-(1,2-dihydroxyethyl)methoxyphenyl] piperazine (II), *cis*-1-[4-{[2-(2,4-dichlorophenyl)-2-(1H-imidazol-1-ylmethyl)-1,3-dioxolan-4-yl]methoxy}phenyl] piperazine (III), and triaconazole, *cis*-1-[4-{[2-(2,4-dichlorophenyl)-2-(1H-1,2,4-triazol-1-ylmethyl)-1,3-dioxolan-4-yl] methoxy}phenyl] -4-(1-methylethyl)piperazine, were supplied by Janssen Pharmaceutica (Beerse, Belgium). The stock ketoconazole solution, 100 μmol/l (53.2 mg/l) in methanol, is stable for at least six months at 4°C. The working ketoconazole 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 ketoconazole), rapidly stir 100 ml of serum and slowly add about 2.7 mg of ketoconazole; continue stirring the mixture for 1 h. Dilute 1 ml to 10 ml with serum and store in 50-μl aliquots at −15°C; this is stable for twenty weeks.

The stock triaconazole internal standard solution, 90 μmol/l (47.9 mg/l) in acetonitrile, is stable for at least six months at 4°C. The working triaconazole internal standard solution, 9 μmol/l in acetonitrile, is prepared freshly on each day of analysis.

Glass vials, 0.3 ml (Pierce Reactivials, Pierce, Rockford, IL, U.S.A.) were obtained complete with screw caps and PTFE-faced discs.

Sample preparation

To a 0.3-ml Reactivial add 20 μl of plasma or serum. At the same time prepare reagent blank, control (QC) and standard vials. In the standard vials place 10, 20, 40, 80 and 150 μl of ketoconazole working solution and evaporate to dryness at 40°C in a stream of dry nitrogen. To the blank and standard add 20 μl of drug-free pooled human serum; to the control add 20 μl of QC serum.

To each vial add 30 μl of working internal standard solution, cap securely, and vortex-mix for 60 sec; centrifuge at 2000 *g* for 1 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). The analytical column is a pre-packed 250 × 4.6 mm I.D. Ultrasphere ODS, average particle size 5 μm, and the guard column is

50 × 4.6 mm I.D. dry-packed with Ultrasphere ODS 20 μm (Beckman Instruments).

The samples are eluted isocratically with a water—methanol—diethylamine (25:75:0.1, v/v) mixture at a constant flow-rate of 1 ml/min. The solution is prepared daily using double-distilled water, filtered (0.45 μm) and degassed before use. With detector sensitivity 0.01—0.005 A.f.s., peak heights at 240 nm are recorded with a 10-mV recorder at a chart speed of 0.25 cm/min.

Inject 20 μl of the supernate into the chromatograph and elute with the mobile phase. Under the above conditions, the retention time for ketoconazole is 9.2 min and for the internal standard 12.9 min. The ratio of peak heights of ketoconazole standard to triaconazole is calculated and the value of QC and unknown specimens calculated by direct proportion.

RESULTS AND DISCUSSION

Fig. 1 shows a chromatogram of blank plasma and a chromatogram of plasma from a patient being treated with ketoconazole. Concentrations as low

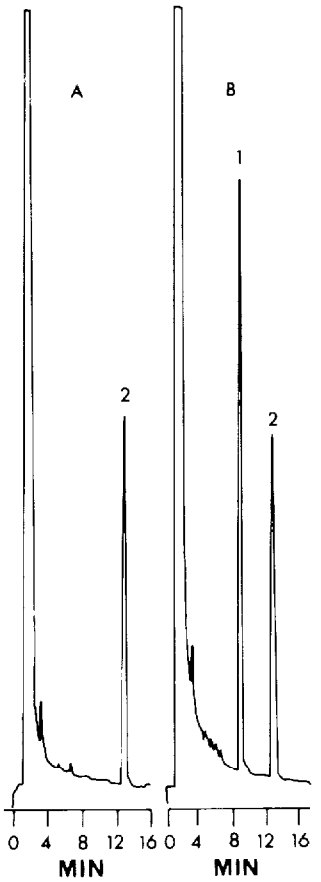

Fig. 1. Chromatograms of (A) extract of drug-free plasma, (B) extract of plasma sample containing 5 μmol/l ketoconazole. Peaks: 1 = ketoconazole; 2 = triaconazole (internal standard).

as 0.1 μmol/l can be measured accurately. Concentration and peak height were linearly related throughout the concentration range investigated, 0.1—20 μmol/l. This adequately covers the range of clinically significant concentrations of the drug in human adult plasma [3, 12—14]; however, in practice in our laboratory, most patient samples were found to contain ketoconazole in the concentration range 0.1—9 μmol/l. Absolute recoveries of ketoconazole at the level 5 μmol/l ranged from 98 to 102%; the correlation coefficient of 0.990 (n = 16) for ketoconazole determinations on paired plasma and serum samples shows that the results are interchangeable. The intra-batch coefficients of variation for replicate 20-μl aliquots of serum (n = 10 in each case) containing 1.0 and 5.0 μmol/l ketoconazole were 4.8% and 3.1%, respectively. Over a period of five months, the between-run coefficient of variation of the assay for samples (n = 21) having a concentration of 5 μmol/l was 4.5%.

The three potential metabolites of ketoconazole (retention times 3.7, 4.8 and 40.1 min, for I, II and III, respectively) were all well resolved from the parent drug and triaconazole. Extracts of plasma samples from patients on griseofulvin, miconazole, nystatin and amphotericin B showed no interfering peaks. Lignocaine (sometimes found in samples as an artefact following its use as a local anaesthetic during blood collection) elutes with ketoconazole; this interference can be eliminated by monitoring the column effluent at 291 nm rather than 240 nm but with a reduction of about eight-fold in sensitivity. The internal standard, triaconazole, is employed as it is a homologue of ketoconazole and, although possessing antifungal activity [15], is not used as a drug. Sample preparation by acetonitrile deproteinization simplifies and speeds the assay; at a 3:2 (v/v) ratio of acetonitrile to serum, the supernate obtained is clear with no micro-precipitate [16]. Techniques previously considered mandatory and designed to either overcome obstruction of the ketoconazole peak by contaminants coming off in the void or quantitatively elute ketoconazole from the column, viz. lengthy extraction procedures [9], the use of Sep-Pak C_{18} cartridges for deproteinization [10], monitoring with a fluorescence detector [11] or the use of a buffer in the mobile phase [9—11] are avoided by using a less polar solvent system and a column with higher theoretical plates. With the procedure described, the later emerging contaminants which Andrews et al. [10] observed when using protein denaturants and which made it necessary for them to extend the individual assay times to 20 min were not evident. The distinct separation of ketoconazole from contaminating peaks allows monitoring of the drug at its maximum absorption at 226 nm when increased sensitivity (0.05—0.1 μmol/l) is required (although with a concomitant increase in baseline drift and noise); even at 240 nm, sensitivities are equal to [9] or in excess of [10, 11] other methods.

The method described is currently being used for pharmacokinetic studies of ketoconazole suspension in neonates and for the therapeutic monitoring of children on oral ketoconazole for severe chronic mucocutaneous candidiasis. Requiring only 20 μl of plasma or serum it offers advantage over other methods in monitoring these paediatric patients.

REFERENCES

1 R.J. Hay, Amer. J. Med., 74 (Suppl. 1B) (1983) 16.
2 R.C. Heel, in H.B. Levine (Editor), Ketoconazole in the Management of Fungal Diseases, Adis Press, Balgowlah, Australia, 1982, p. 67.
3 D. Borelli, J.L. Bran, J. Fuentes, R. Legendre, E. Leiderman, H.B. Levine, A. Restrepo-M. and D.A. Stevens, Postgrad. Med. J., 55 (1979) 657.
4 H.B. Levine and J.M. Cobb, Amer. Rev. Resp. Dis., 118 (1978) 715.
5 B. Dupont and E. Drouhet, Postgrad. Med. J., 55 (1979) 685.
6 R.P. Harvey, R.A. Isenberg and D.A. Stevens, Rev. Infect. Dis., 2 (1980) 559.
7 N.R. Badcock, A. Jarvinen, T. Turner and A.C. Pollard, in preparation.
8 M.A. Pfaller, J.J. Segal and D.J. Krogstad, Antimicrob. Agents Chemother., 22 (1982) 917.
9 K.B. Alton, J. Chromatogr., 221 (1980) 337.
10 F.A. Andrews, L.R. Peterson, W.H. Beggs, D. Crankshaw and G.A. Sarosi, Antimicrob. Agents Chemother., 19 (1981) 110.
11 S.F. Swezey, K.M. Giacomini, A. Abang, C. Brass, D.A. Stevens and T.F. Blaschke, J. Chromatogr., 227 (1982) 510.
12 F.C. Odds, L.J.R. Milne, J.C. Gentles and E.H. Ball, J. Antimicrob. Chemother., 8 (1980) 97.
13 F.C. Odds, Lancet, ii (1982) 771.
14 R.C. Heel, R.N. Brogden, A. Carmine, P.A. Morley, T.M. Speight and G.S. Avery, Drugs, 23 (1982) 1.
15 J.G. Adams, personal communication.
16 S.Y. Chu, L. Oliveras and S. Deyasi, Clin. Chem., 26 (1980) 521.