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Monitoring of fluconazole in serum of patients undergoing hemodiafiltration by solid-phase extraction and high-performance liquid chromatography with ultraviolet detection

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A high-performance liquid chromatographic assay for monitoring the antifungal drug fluconazole in human serum was developed using a C18 reversed-phase column with isocratic elution. The method involved sample clean-up by solid-phase column extraction, and subsequent analysis required only 14 min per sample for separation and quantitation. The assay was precise, with intra- and inter-assay relative standard deviations of $\leq$1.5% and $\leq$3.1%. The minimum detectable concentration of fluconazole was 0.3 nmol/ml. This assay has the advantage of minimizing the risk of interference from co-administered drugs to critically ill patients undergoing hemodiafiltration.

1. Introduction

Fluconazole, 2-(2,4-difluorophenyl)-1,3-bis(1*H*-1,2,4-triazol-1-yl)2-propanol, is an antifungal agent that is a triazole derivative, and has been shown to be effective against a wide range of systemic and superficial fungal infections, following either oral or intravenous administration. This drug has a long half-life and is excreted predominantly unchanged in the urine (Humphrey et al. 1985).

Acute renal failure is a common complication in critically ill patients. Currently, in Japan, the preferred method for blood purification in patients with acute renal failure is continuous hemodiafiltration; this enables better control of fluid balance, better metabolic control, increased hemodynamic stability, and full nutritional support, resulting in a shorter duration of episodes of renal failure and intensive care unit admission. However, continuous hemodiafiltration filters out various antimicrobial drugs through the large pores in the membrane used. It has been reported that continuous hemodiafiltration removes fluconazole from the blood efficiently and at a high rate, resulting in an ineffective fluconazole level in the serum (Kishino et al. 2001). Systemic fungal infections in intensive care unit patients are not frequent. However, their incidence is increasing in concert with high mortality and morbidity, leading to prolonged stays in intensive care unit wards (Vincent et al. 1995; Nucci et al. 1998). It is important therefore, to determine the appropriate use of antifungal agents for systemic antifungal therapy.

The currently available assays for the determination of fluconazole are bioassays (Troke et al. 1985; Van't Wout et al. 1988; Rex et al. 1991), gas chromatographic assays using electron-capture or nitrogen-selective detection (Wood and Tarbit 1986; Debruyne et al. 1988; Harris et al. 1989; Beijnen et al. 1991), and HPLC methods (Hosotsubo et al. 1990; Inagaki et al. 1992; Wallace et al. 1992; Koks et al. 1995; Cociglio et al. 1996; Ng et al. 1996; Majcherczyk et al. 2002). Among the HPLC methods, only one paper relates to the measurement of fluconazole in plasma from patients with acute renal failure undergoing intermittent hemodiafiltration (Cociglio et al. 1996). This method involves sample clean-up by liquid-liquid extraction, and subsequent application to HPLC. The reported retention time of fluconazole was 3.6 min, and the coefficients of variation were 5% between measurements of a single extract injected in duplicate. However, the separation between fluconazole and endogenous substances or co-administered drugs was not satisfactory in our clinical patients on antifungal therapy.

Our purpose was to develop a selective HPLC method with solid-phase extraction for the therapeutic monitoring of fluconazole in serum from patients undergoing hemodiafiltration. This system is suitable for a routine assay of fluconazole to achieve a clear separation from other drugs and serum factors.

2. Investigations, results and discussion

This HPLC-ultraviolet detection system for the measurement of fluconazole was found to be very selective and reliable. The calibration graph for fluconazole injected showed good linearity below 52.24 nmol/ml, and was defined by the equation: $y = (0.7836 \pm 0.086)\,x + (0.0818 \pm 0.0098)$ with a correlation coefficient of 0.9994. The limit of detection (LOD) was 0.3 nmol/ml for fluconazole in our analytical system. The recovery and precision data for fluconazole spiked into drug-free, pooled, human serum are shown in the Table. The recoveries (mean $\pm$ S.D., n = 6) at three different concentrations were higher than 98.3%. The intra- and inter-assay precision expressed as the relative standard deviation (R.S.D.) were $\leq$1.5% and $\leq$3.1%, respectively. Our statistical validation indicates satisfactory performance for the assay method.

Most HPLC methods have involved UV absorbance at 210 nm, in order to increase the sensitivity of assays for the determination of fluconazole (Hosotsubo et al. 1990; Inagaki et al. 1992;

Table: Statistical validation of the determination of fluconazole in human serum

Spiked concentration (nmol/ml)	Intraassay (n = 6)			Interassay (n = 6)		
	Measured concentration mean ± S.D. (nmol/ml)	R.S.D. (%)	Recovery (%)	Measured concentration mean ± S.D. (nmol/ml)	R.S.D. (%)	Recovery (%)
3.27	3.24 ± 0.05	1.5	99.2	3.21 ± 0.10	3.1	98.3
19.59	20.42 ± 0.26	1.3	104.2	19.59 ± 0.48	2.4	100.0
45.71	45.84 ± 0.55	1.2	100.3	45.13 ± 1.16	2.6	98.7

Wallace et al. 1992). However, under such conditions, there is a higher risk of interference from other co-administered drugs or endogenous substances. Fig. 1 shows typical chromatographic patterns for the separation of fluconazole in human serum at 260 nm. The retention time for fluconazole was 7.5 min. The fluconazole peak was well-separated from the other detectable components in human serum, and was not interfered with by other drugs medicated for therapy. However, another published HPLC method for fluconazole assay possesses some advantages such as simplicity, rapidity, and selectivity (Majcherczyk et al. 2002).

Finally, we applied our standard assay to clinical tests. Fig. 2 shows typical profiles of the serum concentration of fluconazole in two patients following the intravenous infusion of 200 mg per day for six days. The serum concentration of fluconazole in one patient on continuous veno-venous hemodialysis reached a level (6 μg/ml) on day 5 that was effective against high-sensitivity *Candida* species. Another patient not undergoing hemodiafiltration had a serum level (10 μg/ml) effective against low-sensitivity *Candida* species at four days.

Because collection of blood samples from seriously ill patients on hemodiafiltration must be approached carefully, a reliable drug assay is required for the clinical laboratory. The HPLC method described here for the measurement of fluconazole in serum is simple, accurate, reliable, and selective. It requires only a single-step extraction using a solid-phase column, and the analytical run time is about 14 min. Our method is suitable for researching the pharmacokinetics of fluconazole, and can also be applied to therapeutic drug monitoring in patients.

3. Experimental

3.1. Chemicals

Fluconazole was provided by Pfizer Pharmaceutical (Tokyo, Japan). Oasis MCX solid-phase extraction cartridges were supplied by Waters (Milford, MA, USA). Acetonitrile and methanol were of chromatographic grade (Wako). Other chemicals and solvents were of analytical reagent grade.

3.2. Chromatographic conditions

Analysis was performed using a Japan Spectroscopic HPLC system consisting of a Model 880-PU pump, 875-UV variable-wavelength detector, 860-CO column oven, 880-50 degasser, 880-02 gradient unit, 802-SC system controller and 807-IT integrator. The system was operated at room temperature at a flow-rate of 1.0 ml/min, employing a Finepak SIL C18S (particle size, 5 μm) reversed-phase column (150 × 4.6 mm i.d.) (Jasco), fitted with a Lichrosorb RP-18 guard gel (15 × 3.2 mm i.d.; particle size, 5 μm) (Cica-Merck). The mobile phase consisted of 25 mM sodium acetate buffer (pH 5.0) and acetonitrile (80 : 20, v/v). Analytes were detected at 260 nm.

3.3. Extraction procedure

Five hundred microliters of serum sample was pipetted into a glass tube. An 8 ml volume of phosphate-buffered saline (PBS) and 80 μl of orthophos-

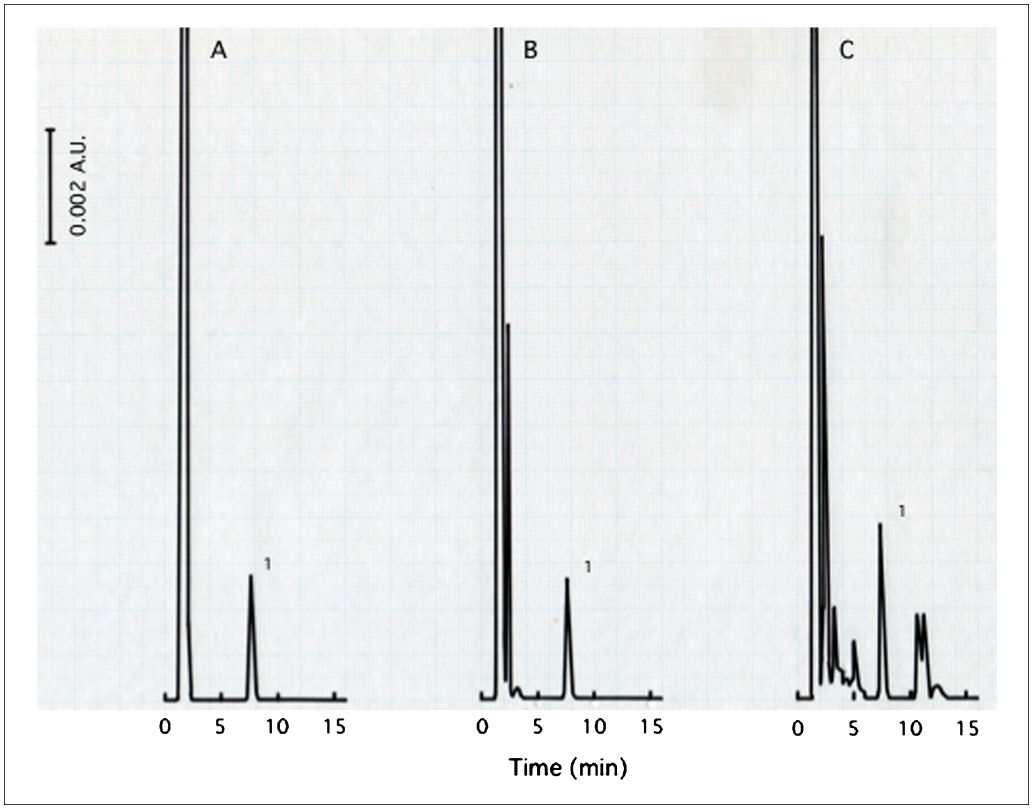

Fig. 1: HPLC elution patterns of fluconazole in human serum. Conditions are described in the Experimental section. Peak 1, fluconazole. (A) Chromatogram of fluconazole in methanol; the peak height of fluconazole corresponds to 9.37 nmol/ml. (B) Chromatogram of an extract from drug-free, pooled, human serum spiked with 9.37 nmol/ml fluconazole. (C) Chromatogram of an extract from serum of patient on continuous hemodiafiltration

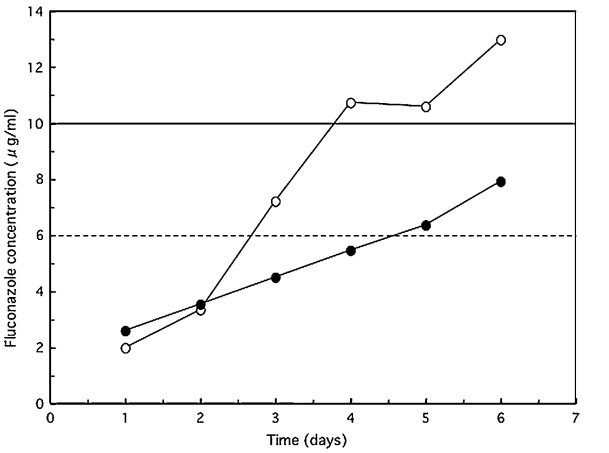

Fig. 2: Serum concentrations of fluconazole in two patients following intravenous administration of 200 mg per day for six days. The horizontal solid line indicates a fluconazole level (10 μg/ml) effective against low-sensitivity *Candida* species, and the horizontal dotted line indicates a level (6 μg/ml) effective against high-sensitivity *Candida* species. (●) = patient on continuous hemodiafiltration, (○) = patient without hemodiafiltration

phoric acid were added to each sample. The tubes were vortex-mixed and samples were allowed to equilibrate for at least 30 min.

Solid phase extraction was performed using Oasis MCX cartridges (3 cm^3/60 mg). These cartridges were conditioned and equilibrated with 1 ml of methanol and 1 ml of water. Each acidified specimen was applied to the cartridge and passed through the bed at a constant flow-rate (1.0 ml/min). Cartridges were sequentially washed with 4 ml of 0.1 M hydrochloric acid, 4 ml of methanol, and, finally, 2 ml of 10% methanol containing 5% ammonium hydroxide. Analytes were eluted with 2 ml of methanol containing 5% ammonium hydroxide, and the clear supernatant obtained was evaporated *in vacuo* using an AES1010 speed-vac concentrator (Savant, Holbrook, NY). The resulting residue was dissolved into 100 μl of the mobile phase, and an aliquot of the mixture was subjected to HPLC analysis.

3.4. Validation parameters

Recovery was assessed by spiking known amounts of fluconazole into drug-free, pooled, human serum. The intra- and inter-assay precision and accuracy were evaluated to ensure reproducibility before actual assay. The intra-assay precision was assessed by analyzing six samples at three concentrations (3.27, 19.59, and 45.71 nmol/ml) for one day. The inter-assay precision was assessed by the same method on six different days.

3.5. Blood sampling and clinical application

We studied two patients receiving daily intravenous infusion of fluconazole for six days. Informed consent was obtained before therapy began, and our study was approved by the hospital's ethical committee. One patient (male, 70 years old) with external traffic injuries was not undergoing hemodiafiltration. The other patient (male, 89 years old) with an acute abdominal condition was undergoing hemodiafiltration. Both were receiving two other medications (imipenem/cilastatin and vancomycin) during the research period.

A blood sample (3 ml) was collected at 3 h after intravenous administration of 200 mg fluconazole, and all serum samples were stored frozen at −80 °C prior to analysis.

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