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High-performance liquid chromatographic determination of fluconazole in plasma

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Abstract

A high-performance liquid chromatographic method with ultraviolet absorbance detection at 260 nm was developed for the analysis of fluconazole in plasma. The method involves sample clean-up by liquid–liquid extraction. The proposed technique is reproducible, selective, reliable and sensitive. Calibration standards were prepared in the range 1.25–20 mg/l. The limit of quantitation was 0.4 mg/l. The coefficients of variation were 5% between measurements of a single extract injected in duplicate, and 7% between two extractions of spiked samples at the same concentrations. The separation between fluconazole and endogenous substances was satisfactory. This method was designed in order to minimise the risk of interference from substances that could be co-administered to critically ill patients undergoing hemodiafiltration. With a run time below 5 min, the present method is rapid and easy to use for later clinical studies, as well as for routine monitoring.

Keywords: Fluconazole

1. Introduction

Fluconazole, 2-(2,4-difluorophenyl)-1,3-bis(1H-1,2,4-triazol-1-yl)-2-propanol (Fig. 1), is a synthetic triazole derivative antifungal agent that has been shown to be effective against a wide range of systemic and superficial fungal infections, following both oral and intravenous administrations. While it is generally not necessary to measure fluconazole levels, its dose must be adjusted in patients with renal insufficiency [1].

Preliminary studies to quantify this drug in biological fluids involved gas chromatography [2–5] and high-performance liquid chromatography (HPLC) [6–8]. All of them used tedious extraction or solid-phase extraction [9] purification steps and sometimes evaporation under a stream of nitrogen to

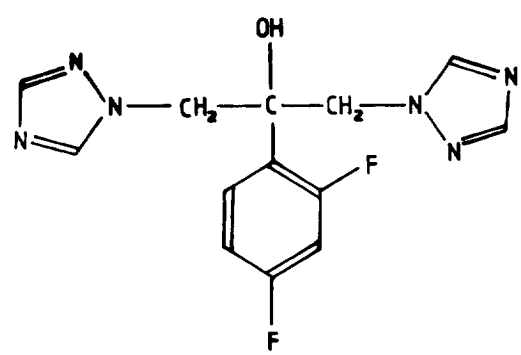

Fig. 1. Structural formula of fluconazole.

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increase the sensitivity, in order to conduct pharmacokinetic studies at the effective dose level.

The purpose of this study was to develop a rapid, sensitive and selective method for the determination of fluconazole in plasma. This method was validated according to Good Laboratory Practice guidelines for use in drug monitoring and for dose adjustment in patients with acute renal failure undergoing intermittent hemodiafiltration.

2. Experimental

2.1. Reagents

The stock solution contained fluconazole at a concentration of 2 mg/ml (Triflucan, Pfizer, Orsay, France). Double-distilled deionised water, obtained by passing water through a Milli-Q reagent water system (Millipore, Saint Quentin/Yvelines, France), was used. Acetonitrile was of LiChrosolv grade (Merck, Darmstadt, Germany) and used without further purification. Glacial acetic acid, sodium carbonate and ammonium hydroxide were of all analytical grade (Merck). The buffer consisted of acetic acid (25 m*M*) in distilled water and was adjusted to pH 4.0, using ammonia. The validation samples were prepared in pooled human plasma samples obtained from healthy subjects.

2.2. Instrumentation

The isocratic system consisted of the following components: a Model SP 8810 pump and a Model Spectra 100 variable-wavelength detector (Thermo Separation Products, Les Ulis, France) and a Model 655A-40 autosampler equipped with a 100-μl loop and a D2000 chromato-integrator (Merck).

2.3. Chromatographic conditions

Isocratic separation was achieved using an Ultrabase C_8 column (125×4 mm I.D., 5 μm particle size) supplied by SFCC (Neuilly Plaisance, France). The mobile phase, consisting of distilled water–acetonitrile (72:28, v/v), was pumped at a flow-rate of 1.0 ml/min. The analytes were detected at 260 nm using a setting of 0.005 AUFS. The injected volume was 40 μl. Chromatography was performed at ambient temperature (20±2°C).

2.4. Sample extraction

A demixing method was used [10]. To one volume (500 μl) of plasma or distilled water, one volume of acetonitrile containing fluconazole at 5 mg/l and an excess of sodium carbonate were added and the mixture was vortex-mixed for 10 s. After centrifugation (10 min at 2000 *g*), 350 μl of the supernatant were withdrawn and diluted with 700 μl of distilled water, in order to obtain the same acetonitrile–water proportion as in the mobile phase.

2.5. Standard curve

Calibration standards were prepared using concentrations of 1.25, 2.5, 5, 10 and 20 mg/l of fluconazole in human plasma and distilled water. Standard samples were prepared by adding appropriate volumes of fluconazole solution. The volume added was always smaller than or equal to 2% of the total volume of the sample, so that the integrity of the sample was maintained. These samples were treated as indicated above.

2.6. Recovery

The recoveries of water extracts and plasma extracts were estimated as the ratio of average peak heights of extracted samples to the average peak heights of unextracted samples.

2.7. Validation design and analysis of variance (ANOVA)

The peak height of fluconazole was used as the assay parameter. Peak heights were measured in extracts prepared from water solutions and from human plasma spiked with fluconazole at five concentrations (each extract contained the standardised addition of 5 mg/l of fluconazole). A set of duplicates of blank plasma and water samples provided

the mean measurement value of standardised addition. Similarly, peak heights were measured from unextracted aqueous solutions at the same concentrations. All determinations were performed in duplicate. This experimental design was replicated on two successive days.

Precision of the method was assessed with a previously published, mixed experimental design [11]. The design factors were drug concentrations (five) and sample matrix (two levels: water and human plasma). Each sample was extracted twice and each extract was injected twice, according to the nested within-cell pattern, in order to estimate separately the variances of chromatographic measurement and of the extraction procedure. All ANOVA calculations were made from Napierian logarithm of peak height corrected from the mean measurement of the standardised addition, as follows.

(1) Homoscedasticity was checked through Bartlett's test [12] of all measurements and of ln (measurement) duplicates. In the particular case of duplicates, the C term in Bartlett's test simplifies down to: $k^* \Sigma(\ln(\Delta i^2)) - \Sigma(\ln(\Delta i^2)) - k^* \ln(k)$, Δi being the difference between the two measurements (or log-measurements) from the same ith extract, and k being the number of duplicates submitted to the test.

(2) Following factorial analysis, F tests were applied to the extraction difference between water and plasma (two matrices, one degree of freedom), and to linear regression components (regression common to both matrices, common non-linearity and second-order curvature); the concentration–matrix interaction term was split into non-parallelism and opposite curvature of regression lines. The denominator of F tests was the within-cell mean square. The regression coefficient b (slope of the bilogarithmic regression line) was compared to the theoretical value of one using Student's t test of $(b-1)/s_b$, s_b^2 being calculated as usual as the within-cell mean square divided by the sum of squares of ln (concentration).

(3) The within-cell mean square was split into measurement (within-extract) variance estimate, Var_m, and variance of extract pairs, Var_{2x}. Variance of extraction was calculated as $Var_x = Var_{2x}/2$. The coefficients of variation (C.V.) of chromatographic measurements and of the extraction step were calculated as the respective square roots.

2.8. Limit of quantitation (LOQ)

The LOQ was determined from the peak and the standard deviation of the signal-to-noise ratio (S/N). The LOQ was defined as the sample concentration of fluconazole resulting in a peak height of eight times S/N.

The LOD was defined as the sample concentration of fluconazole resulting in a peak height of three times S/N.

2.9. Specificity

To evaluate the specificity of the method, 0.5 ml of drug-free plasma was carried through the assay procedure and the retention times of endogenous compounds in plasma were compared with those of fluconazole.

Specificity of the method was assessed to test matrix influence between different plasma samples from hospitalised patients ($n=14$).

The interferences from other drugs that could be co-administered in an intensive care unit were also studied. The following drugs were checked: amikacin, tobramycin, ofloxacin, vancomycin, amoxicillin–clavulanic acid, piperacillin, teicoplanin, imipenem–cilastatine, theophylline, methotrexate, carbamazepine and its metabolite (epoxycarbamazepine), valproic acid, phenytoin, phenobarbital, thiopental, midazolam and flunitrazepam.

2.10. Stability study

In the present study, the stability of fluconazole in plasma was assessed from spiked samples (1.25, 5 and 20 mg/l), after bench-top storage at room temperature for 8 h, at +4°C for 48 h and at −20°C for one month. They were analysed immediately after preparation and after storage. Prior to the analysis of samples after cold storage, they were brought to room temperature (20°C) and vortex-mixed well. Each determination was performed in duplicate.

3. Results

3.1. Retention times

The observed retention time was 3.6 min and the corresponding capacity factor was 3.5 (Fig. 2). The resolution between the peak of fluconazole and the last peak was 1.4.

3.2. Linearity and precision.

Calibration graphs and the distribution of corresponding residuals are shown in Fig. 3. The results summarised in Table 1 show that the calibration relationship of the present assay agrees with the regression model: $\ln S = \ln a + \ln C$, equivalent to $S = a \cdot C^1$: Peak height measurements may be considered proportional to concentrations, which agrees with a linear model without an intercept.

ANOVA results of validation design presented in Table 1 showed that C.V.s were 5.2% between measurements of a single extract injected in duplicate and 7.0% between two extractions of spiked samples at the same concentrations. The common bilogarithmic regression slope of 1.01 did not differ significantly from the target value of one ($t_{60} < 1$); curvature common to both matrices and opposite curvature in either were not significant.

Interestingly, there was no significant difference in peak heights between the two matrices, which means that standardisation is possible with aqueous solutions of fluconazole.

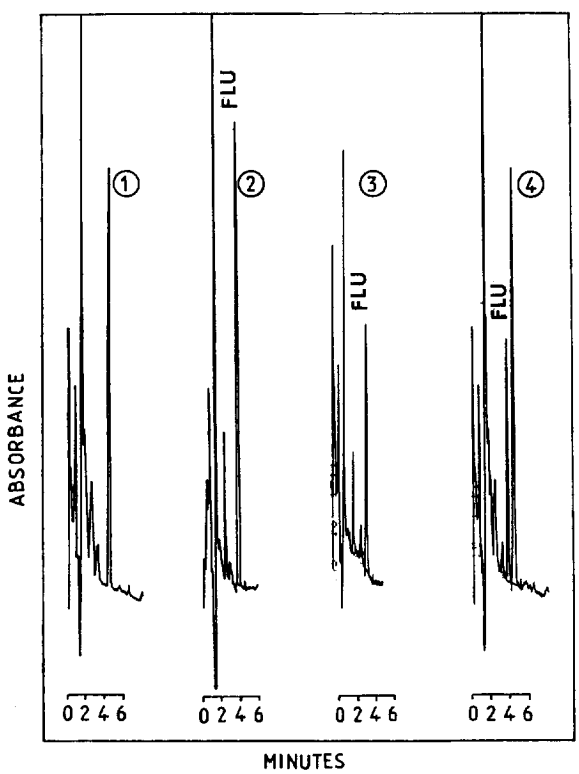

Fig. 2. Chromatograms of blank plasma (1), aqueous solution (10 mg/l) (2), aqueous (3) and plasma (4) extracts spiked with 10 mg/l of fluconazole. For chromatographic conditions, see Section 2.3.

3.3. Limit of quantitation and limit of detection

The limit of quantitation was 0.4 mg/l, the corresponding coefficient of variation ($n=6$) was 2.3%. At this level, the absolute relative error averaged 6%.

The limit of detection was 0.15 mg/l.

3.4. Specificity and recovery

No endogenous substance interfered at the retention time of fluconazole.

From random plasma samples ($n=14$ patients), no interference of the matrix was observed. The seven plasma samples containing the standardised addition of 5 mg/l of fluconazole give a C.V. value of 5.8%. While the seven plasma samples spiked with 10 mg/l of fluconazole give a C.V. of 4.8%. The absolute relative error is always lower than 3.1%.

No interference was found when all of the drugs that could be co-administered were tested.

The recovery averaged 97% ($n=10$).

3.5. Stability

Fluconazole was stable in plasma for 8 h at room temperature, 48 h in a refrigerator (2–8°C) and for one month at −20°C. No significant deviation was found from the nominal values.

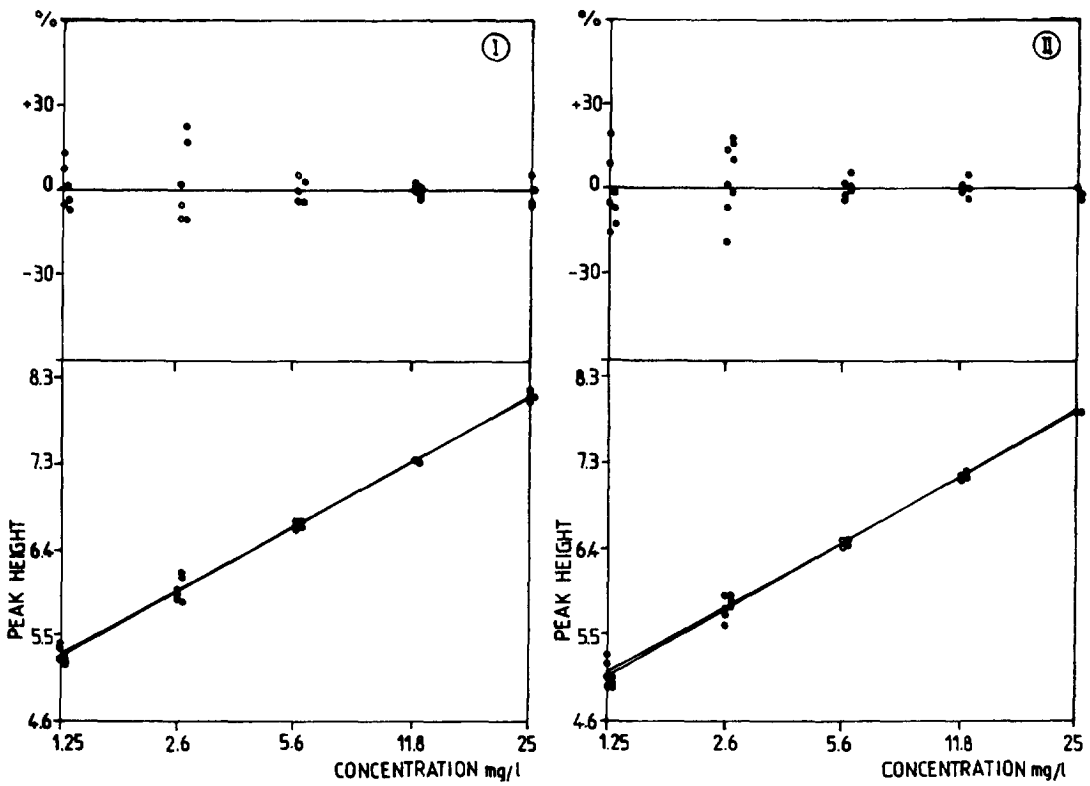

Fig. 3. Bi-logarithmic calibration graphs and distribution of residuals of fluconazole on days I and II. (●) spiked plasma; (○) spiked water.

Table 1
ANOVA of fluconazole peak height (Napierian logarithms)

Components	df	SSq	MSq	SD_{ln} (C.V.)	Tests[a]	Others
Total	79	82.3014617				
Between cells	19	81.9123615				
Between days	1	1.33940423				
Between matrices	1	0.01476122			$F(1/60)<1$	$\Delta=1.7\%$
Linear regression	1	80.3082507		$s_b=0.0367$	$t_{60}<1$	$b=1.007$
1st curvature	1	0.00203607			$F(1/60)<1$	
Sigmoidicity	1	0.00027423			$F(1/60)<1$	
Non-parallelism	1	0.0344432			$F(1/60)<1$	
Opposite curvature	1	0.00028745			$F(1/60)<1$	
Others	3	0.0123733	0.00412443			
Day interaction	9	0.20053103	0.02228123			
Within cells	60	0.38910023	0.006485	0.08052952	denominator	
Between measures	40	0.1452029	0.00363007	0.06025008		
Extract pairs	20	0.24389733	0.01219487			
Extraction			0.00609743	0.07808606		

df=degrees of freedom; SSq=sum of squares; MSq=mean squares; SD_{ln} (C.V.)=logarithmic standard deviation, i.e. coefficient of variation of arithmetic values (see text); Δ=percent mean difference (plasma extracts − water extracts)/water extracts; b=regression slope; s_b=standard deviation of the regression slope.

[a] None of the tests were significant.

4. Discussion

The validation experiment was designed to cover about a twenty-fold range of concentrations, which is actually obtained with five concentrations in geometrical progression of ratio (dilution factor) 2.

In previous reports [10,11], we have already shown that the logarithmic transformation currently generates values closer to homoscedasticity than the untransformed measurements. Thus, linearity, precision and matrix effects were assessed in the validation design after logarithmic transformation of HPLC measurements. Logarithmic transforms appear more suitable than arithmetic values for several reasons. Firstly, the often neglected requirement of homoscedasticity for ANOVA is currently better fulfilled. Secondly, analytical non-linearity (which means departure from direct proportionality) can be assessed rather simply by testing non-linearity of the logarithmic regression line together with departure of the logarithmic slope from the theoretical value of one. Finally, additive–subtractive statistical comparisons are replaced by the more familiar scale-free relative ("percent") comparisons, e.g., standard deviations by coefficients of variation: as already pointed out [10], Napierian logarithm standard deviations directly provide approximations to less than 10% of the corresponding C.V.s, if C.V.s are smaller than 20% [13].

In reference to gas chromatographic published methods, the bioassay [14] performs quite well but is time-consuming. Most published methods for quantifying fluconazole in body fluids used liquid–liquid extraction and sometimes evaporation of the organic extract under nitrogen stream. Some of them included back-extractions from the organic phase into acidic solution [2,6,14]. Most HPLC methods involved UV absorbance at 210 nm, in order to increase the sensitivity of the assay [4,8,15]. However, under such conditions, there are more risks of interferences by other co-administered drugs or endogenous substances. The present HPLC method with UV absorbance detection provides a rapid assay to determine fluconazole in plasma and aqueous matrices, such as dialysates. We have validated this method for concentrations ranging from 1.25 to 20 mg/l (although the limit of detection of 0.15 mg/l, was clearly lower); indeed, concentrations of 8 to 9 mg/l [5] were currently considered to represent the clinically relevant range for fluconazole during treatment of candidasis. This method, including demixing extraction, was designed in order to minimise the risk of measuring interference substances that have retention times close to that of fluconazole and that could be co-administered to critically ill patients and that cannot be exhaustively investigated. A simple precipitation of proteins by an equal volume of acetonitrile has been proposed by Hosotsubo et al. [15], but this method did not offer enough sensitivity and selectivity. Since the actual retention time of fluconazole in the proposed method is determined by the amount added, any interfering substance will be easily detectable by a widening of the peak or by the presence of a shoulder on the peak. However, the amount added is likely to decrease precision and sensitivity. Nevertheless, the method showed good reproducibility, accuracy and sufficient sensitivity. It is as sensitive and accurate as gas chromatography [2–4] and the limit of detection is 0.15 mg/l. The separation between fluconazole and endogenous substances (including potential metabolites) was satisfactory. Moreover, with a run time of 5 min, this method is rapid and easy to use for routine clinical monitoring.

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