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Short Communication

Gas chromatographic analysis, with electron capture detection, of the antifungal drug fluconazole in microvolumes of human plasma*

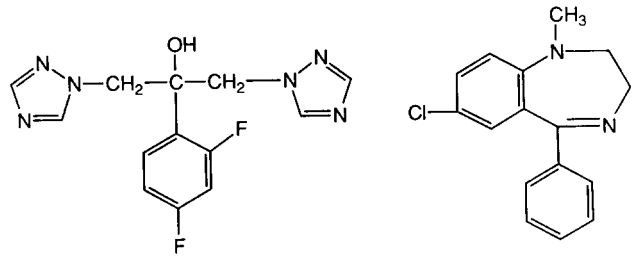
J.H. BEIJNEN,†‡ P.L. MEENHORST,§ R. VAN GIJN,‡ W.A. HAZELAGER,‡ C.H.W. KOKS‡ and W.J.M. UNDERBERG‖

‡*Department of Pharmacy, Slotervaart Hospital/Netherlands Cancer Institute, Louwesweg 6, 1066 EC Amsterdam, The Netherlands*
§*Department of Internal Medicine, Slotervaart Hospital, Louwesweg 6, 1066 EC Amsterdam, The Netherlands*
‖*Department of Pharmaceutical Analysis, Faculty of Pharmacy, State University of Utrecht, P.O. Box 80.082, 3508 TD Utrecht, The Netherlands*

Keywords: *Fluconazole; plasma assay; gas chromatography; electron capture detection.*

Introduction

Fluconazole (Fig. 1) is a bis-triazole derivative with profound antifungal activity. The drug is used in the treatment of vaginal, oropharyngeal and oesophageal candidiasis [1]. Fluconazole is a particularly promising treatment in patients with Acquired Immuno Deficiency Syndrome (AIDS)-related cryptococcal meningitis [2]. The bioavailability after oral administration exceeds 90% and the drug is eliminated, predominantly unchanged, by renal excretion. The protein binding is 12%. Pharmacokinetic data on fluconazole in patients infected with human immunodeficiency virus (HIV) are scarce but are essential, in view of its therapeutic application. Knowledge of the drug's penetration into cerebrospinal fluid, saliva and sputum is important to get a full picture of the relationship between *in vivo* drug disposition and therapeutic efficacy. In the literature there are two GC methods described for the bioanalysis of fluconazole [3, 4]. The first method was described by Wood and Tarbit [3]. This GC assay with electron capture detection is very laborious and includes a triple extraction procedure and extensive pre-treatment of the packed column because of adsorption of the analytes. The other reported GC method, utilizing selective nitrogen detection, is less time-consuming but also less sensitive [4]. In both GC methods an internal standard,



I II

Figure 1
Structures of fluconazole (I) and the external standard medazepam (II).

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†Author to whom correspondence should be addressed.

encoded UK-47,265, is used, which is not commercially available.

The purpose of this study was to develop an alternative, simple, sensitive GC method using only a minimal amount of plasma and a single extraction step. The proposed methodology can be used for routine analysis and pharmacokinetic research in, for example, HIV infected patients.

Experimental

Chemicals

Fluconazole originated from Pfizer BV (Rotterdam, The Netherlands). Medazepam was obtained from Roche Nederland BV (Mijdrecht, The Netherlands). All other chemicals and reagents were of analytical grade.

Sample preparation

To 100 μl of plasma (unknown sample or calibration samples spiked with 2–20 μl of a fluconazole solution in water with concentration 0.5 μg ml^{-1} or 5 μg ml^{-1}), 50 μl of 1 M sodium hydroxide solution and 1.0 ml of chloroform are added. After vortex mixing for 30 s and centrifugation at 12 000 rpm for 5 min, the aqueous supernatant and protein interface are removed by suction under reduced pressure. Next, 0.5 ml of the resulting clear organic extract is transferred to a clean vial and evaporated to dryness under a gentle stream of nitrogen at 40°C. Then, 500 μl of methanol is added and, again, it is evaporated to dryness under a nitrogen stream. This procedure is repeated with 200 μl of methanol. The resulting residue is dissolved in 25 μl of methanol containing the external standard medazepam (concentration: 25 μg ml^{-1}) for the samples in the range 100–1000 ng ml^{-1} or the residue is dissolved in 10 μl of methanol containing medazepam (concentration: 2.5 μg ml^{-1}) in the concentration range 10–100 ng ml^{-1}; 1 μl of the resulting solution is injected into the chromatograph.

Gas chromatography

A HP-5830 A gas chromatograph coupled with a HP 18803 electron capture detection system and a HP 18850 GC terminal with integration facilities (all from Hewlett–Packard, Avondale, PA, USA) were used. The analysis were performed on a semi-capillary HP 1-methylsilicone gum column (10 m long; i.d. 0.53 mm). The carrier gas was a mixture of argon and methane (95:5, v/v) with a flow rate of 5 ml min^{-1}. Other chromatographic conditions are: injector temperature, 250°C; oven temperature, 220°C; detector temperature, 300°C; make-up gas: argon + methane (95:5, v/v) with flow 35 ml min^{-1}.

Validation

The method was validated in terms of linearity, recovery and precision by assaying repeatedly spiked, drug-free plasma samples with known concentrations of fluconazole. Spiked samples were also stored at 60°C for 30 min to check whether the analyte is stable under conditions leading to inactivation of HIV.

Results and Discussion

The presence of two fluorine atoms and six nitrogen atoms in the fluconazole molecule (Fig. 1) offer the possibility to use an electron capture or nitrogen detection system coupled to the GC. Comparison of both detection systems revealed that the electron detection mode is 100 times more sensitive than the nitrogen detector. A single liquid–liquid extraction step for the isolation of fluconazole from plasma has been investigated with ethyl acetate, cyclohexane and chloroform as sample pre-treatment. Chloroform gave the best results in terms of recoveries and selectivity. However, it is an unusual extraction fluid when analysis with GC and electron capture detection follows because of disturbances of the detection system with high background signals. But the repetitive dissolution steps of the residue with methanol, after plasma extraction with chloroform, resulted in an effective removal of traces of chloroform with no interference in the following GC analysis.

Medazepam was used as the external standard of the assay but was added in the final 25 μl methanol because it is volatile under the evaporation conditions used. The validation parameters of linearity, precision and accuracy are listed in Table 1 and demonstrate that the proposed methodology give reliable results. The recoveries of fluconazole were tested at plasma drug concentrations of 500 ng ml^{-1} and 50 ng ml^{-1}. The results were: 90.1 ± 4.2% (n = 4) and 99.9 ± 8.8% (n = 4), respectively. The detection limit of the assay is 3 ng ml^{-1} using a 100 μl plasma sample. The stabil-

Table 1
Equations of calibration lines, accuracy and precision for the bio-analysis of fluconazole

Concentration range (ng ml^{-1})	Equation	r^2	n
10–100	$y^1 = 0.423\ (\pm 0.063) + 0.0124\ (\pm 0.0008)x$	0.9898	8
100–1000	$y^2 = 0.0032\ (\pm 0.0031) + 0.00024\ (\pm 0.000003)x$	0.9991	8

Theoretical conc. (ng ml^{-1})	Measured conc. (ng ml^{-1})	Accuracy (%)	RSD* (%)	n
49.4	2	3	4	5
45.2				
91.5				
9.2				
4				
494.0	489.3	99.1	4.8	4

y^1 is the peak height ratio between fluconazole and the external standard medazepam, y^2 is the peak area ratio between fluconazole and medazepam and x is the fluconazole concentration in ng ml^{-1}.
*RSD: relative standard deviation.

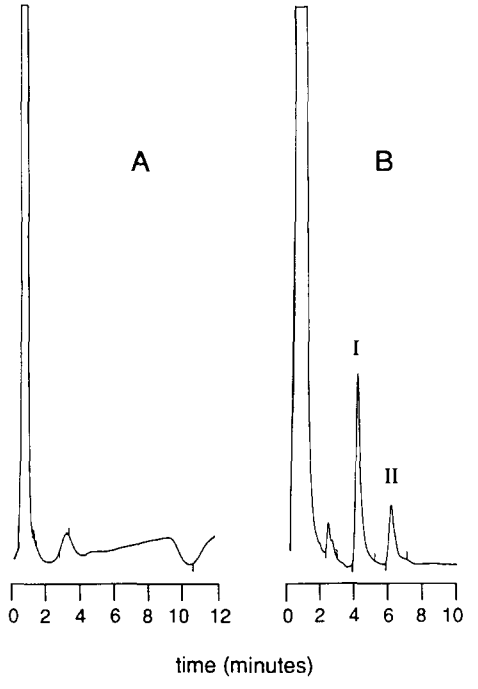

Figure 2
GC chromatograms of blank plasma sample (A) and a patient sample (B) (fluconazole concentration: 530 ng ml^{-1}). I: fluconazole; II: medazepam.

ity of fluconazole was investigated during incubation at 60°C for 30 min. This procedure inactivates HIV [5]. The experiments were executed at a drug concentration of 25 ng ml^{-1} and revealed that fluconazole is stable under these experimental conditions. Representative chromatograms are shown in Fig. 2.

In conclusion, a simple, rapid and sensitive GC method is presented for the bioanalysis of fluconazole, which requires only a 100 µl plasma sample per time point. The method is used successfully in our laboratory for pharmacokinetic research in HIV infected patients.

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