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# High-performance liquid chromatographic determination of the antifungal drug fluconazole in plasma and saliva of human immunodeficiency virus-infected patients

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**Abstract**

A high-performance liquid chromatographic (HPLC) assay has been developed for the determination of the antifungal drug fluconazole in saliva and plasma of patients infected with the human immunodeficiency virus (HIV). Samples can be heated at 60°C for 30 min to inactivate the virus without loss of the analyte. The sample pretreatment involves a liquid–liquid extraction with chloroform–1-propanol (4:1, v/v). The chromatographic analysis is performed on a Lichrosorb RP-18 (5 μm) column by isocratic elution with a mobile phase of 0.01 *M* acetate buffer (pH 5.0)–methanol (70:30, v/v) and ultraviolet (UV) detection at 261 nm. The lower limit of is 100 ng/ml in plasma (using 500-μl samples) and 1 μg/ml in saliva (using 250-μl samples) and the method is linear up to 100 μg/ml in plasma and saliva. At a concentration of 5 μg/ml the within-day and between-day precision in plasma are 7.1 and 5.7%, respectively. In saliva the within-day and between-day precision is 10.8% (at 5 μg/ml). The methodology is now being used in pharmacokinetic studies in HIV-infected patients in our hospital.

## 1. Introduction

Fluconazole is an orally active bis-triazole antifungal agent, which is used in the treatment of superficial and systemic candidiasis and in the treatment of cryptococcal infections in patients with the acquired immuno deficiency syndrome (AIDS).

The bioavailability after oral administration exceeds 90%. The absorption is pH independent and the drug is eliminated, predominantly unchanged, by renal excretion. As a result of its beneficial lipophilic/hydrophilic profile and its low (12%) binding to plasma proteins, fluconazole readily penetrates into body tissue [1]. Fluconazole can be given orally or intravenously and is usually well tolerated, with no major adverse reactions. Because of this favourable profile, fluconazole is increasingly used in the treatment of oropharyngeal candidosis in patients with AIDS. Data on fluconazole pharmacokinetics in saliva of patients infected with human immunodeficiency virus (HIV) are absent but important in view of its therapeutic application. Knowledge of the penetration of the drug in saliva is necessary to get a better picture of the

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relationship between in-vivo drug disposition and therapeutic efficacy. Also the influence of patient-related variables (e.g. the frequently encountered complaint of a "dry mouth" in AIDS-patients/"sicca syndrome") on saliva concentrations can be studied, which may have practical implications in therapeutic decision making. Furthermore, if a close correlation between fluconazole concentrations in saliva and plasma should exist, drug monitoring in saliva, as a non-invasive method, will be preferable because of the reduced risk of disease transmission.

The currently available assays for fluconazole are bioassays [2–4], gas chromatographic (GC) assays using electron-capture or nitrogen-selective detection [5–7] and high-performance liquid chromatographic (HPLC) methods [8–11]. Since there is a discrepancy between in-vitro and in-vivo antifungal activity of fluconazole and because medium conditions are known to influence the in-vitro activity of fluconazole, the bioassay is not a very reliable method. Although the bioassay has been improved (with regard to between-run variability) by Rex et al. [4], it remains a time-consuming method. The GC methods are very sensitive, but the sample preparation is sometimes laborious [5] and the validated concentration range is too narrow for use in saliva and plasma samples [6,7]. Furthermore the reported GC methods are more sensitive to interferences. The published HPLC methods also have their limitations. They all use an experimental drug instead of a commercially available substance as internal standard. Most methods [9–11] monitor for UV absorption at a wavelength of 210 nm, which increases the sensitivity, but also makes the method more vulnerable for interference by other drugs or endogenous substances. Moreover these methods have not been validated for saliva as biological matrix.

In our hospital a research program, focussing on the treatment of oropharyngeal candidosis and the pharmacokinetics of the employed antifungals, has been initiated. Therefore we developed a HPLC method for the quantitation of fluconazole in saliva and plasma. This method has been fully validated, uses a single-step extraction and phenacetin as internal standard for plasma samples (Fig. 1). The assay appeared to be very robust and sufficiently sensitive for drug level monitoring with UV detection at 261 nm.

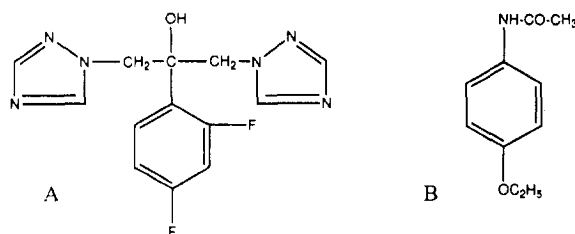


Fig. 1. Chemical structures of (A) fluconazole and (B) internal standard phenacetin.

## 2. Experimental

### 2.1. Equipment

The HPLC system consisted of a Type 510 solvent-delivery system (Waters Assoc., Milford, MA, USA), a Spectra 200 programmable wavelength detector (Spectra Physics, Santa Clara, CA, USA), an SP 8880 automatic sample injection device and a SP 4600 integrator (both Spectra Physics). The analytical column was a Lichrosorb RP-18 column (125 × 4 mm I.D.; particle size 5 $\mu$m) (Merck, Darmstadt, Germany) protected by a LiChroCART 4-4 (4 × 4 mm I.D.) pre-column packed with LiChrospher 100 RP-8 (5 $\mu$m) material (Merck). UV spectra of fluconazole were recorded with a SP8-400 UV-Vis spectrophotometer (Pye Unicam, Cambridge, UK).

### 2.2. Chemicals

Fluconazole was kindly provided by Pfizer Central Research (Sandwich, UK). Chloroform and methanol were obtained from Promochem (Wesel, Germany). Sodium hydroxide, sodium acetate, phenacetin, 1-propanol and hydrochloric acid (37%) were all analytical grade and purchased from Merck. Home-made distilled water was used throughout. Drug-free heparinized

human plasma was obtained from the Central Laboratory of the Blood Transfusion Service (Amsterdam, Netherlands). Drug-free saliva was obtained from healthy volunteers.

### 2.3. Stock solutions

A stock solution of fluconazole (1mg/ml) was prepared by dissolving the appropriate amount of the drug, accurately weighed, in methanol. Typical amounts of the stock solution were diluted with methanol to give reference solutions with concentrations of 500, 50, 5 and 0.5 μg/ml of fluconazole. The solutions were stored, prior to analysis, at −30°C and were found to be stable for at least four weeks under these condition. A stock solution of the internal standard phenacetin (20 μg/ml) was freshly prepared on each day of analysis by dissolving the appropriate amount of this drug in methanol.

### 2.4. Calibration samples

*Plasma*

To achieve fluconazole calibration concentrations of 0.1, 0.2, 1, 2, 10, 20 and 100 μg/ml appropriate amounts of the various diluted stock solutions were added to 10-ml polypropylene tubes (International Medical Products, Zutphen, Netherlands). To each tube 100 μl of the stock solution of phenacetin (20 μg/ml) was added. The solutions were evaporated to dryness under a nitrogen stream at 40°C. Next, 500 μl of drug-free plasma was added to each tube and the tubes were mixed on a vortex-mixer for 10 s. This mixing was repeated after adding 50 μl of 5 *M* NaOH to the tubes. Next, 5.00 ml of extraction fluid (chloroform–1-propanol; 4:1, v/v) was added and the tubes were shaken for 15 min and subsequently centrifuged for 15 min at 2500 *g*. The organic extract was then transferred into a clean polypropylene tube and evaporated to dryness under a nitrogen stream at 50°C. The residues were redissolved in 60 μl of methanol, vortex-mixed for 30 s, and 140 μl of 0.01 *M* sodium acetate buffer (pH 5.0), vortex-mixed for 30 s and then transferred into polypropylene Eppendorf cups and centrifuged for 5 min at 1000 *g*. The supernatants were transferred into autosampler vials and 100 μl aliquots were injected onto the HPLC system.

*Saliva*

The calibration standards in saliva were prepared in the same way as in plasma, with the following adaptations : 250 μl of drug-free saliva was used instead of 500 μl of plasma; because of too large variations (coefficient of variation 21%, $n = 10$) in the extraction recoveries of phenacetin from saliva no internal standard was used, so, after shaking and centrifugation an exact (4.00 ml) amount of the extraction mixture was transferred to the clean polypropylene tubes.

### 2.5. Clinical samples

Blood samples of patients were collected in heparinized tubes. Plasma was immediately isolated by centrifugation and subsequently incubated at 60°C for 30 min to inactivate the HIV [12]. After this procedure the samples were stored at −30°C prior to analysis. Stimulated mixed saliva samples were obtained in a saliva collecting device (Salivette, Sarstedt, Nümbrecht, Germany) by chewing for 30–45 s on cotton wool swabs impregnated with 20 mg of citric acid. Saliva was separated from the swabs by centrifugation and stored at −30°C prior to analysis.

### 2.6. Chromatography

Chromatographic analysis was performed at ambient temperature with a mobile phase of 0.01 *M* sodium acetate buffer (pH 5.0)–methanol (70:30, v/v). The acetate buffer was adjusted to pH 5.0 with hydrochloric acid (37%). Prior to use, the mobile phase was filtered under reduced pressure through a 0.2-μm cellulose acetate filter (Sartorius, Spruyt–Hillen, Utrecht, Netherlands). The absorbance was monitored at 261 nm, which corresponds with a UV absorption maximum of fluconazole. The flow-rate was maintained at 1.0 ml/min and aliquots of 100 μl were injected onto the chromatograph.

### 2.7. Validation parameters

*Calibration curves*

All standards (both in plasma and saliva) were extracted and analyzed in duplicate. Linear regression (response versus concentration, $y$–$x$), weighted linear regression ($y$–$x$ with weighting factor $1/x$ and $1/x^2$) and a power fit model ($\log y$–$\log x$) were applied to the analytical results. These response functions were investigated by calculating correlation coefficients and percent relative concentration residuals (%RCR) of the analyzed results. %RCR is defined as %RCR = 100 (IC − NC)/NC, where IC and NC represent the interpolated and nominal concentration, respectively [13].

*Recovery*

Recovery of fluconazole from plasma and saliva was calculated by comparing the slopes of the $y$–$x$ curves of the standards in plasma and saliva with the slope of the same standards prepared in eluent.

*Lower limit of quantitation*

The lower limit of quantitation is defined as the concentration of the lowest standard in the analytical run which is quantified with a deviation of the actual concentration and a coefficient of variation for precision of less than 20%.

*Accuracy and precision*

The accuracy and precision (between-day and within-day) of the method were determined on three different occasions by replicate analyses of three known concentrations, equally divided over the calibration curve. The parameters were calculated using one way analysis of variance (ANOVA).

*Selectivity*

All medication used by the patients whose samples were analyzed was registered. The possible interference of the following drugs were investigated: zidovudine, didanosine, ganciclovir, foscarnet, amphotericin B, nystatin, itraconazole, co-trimoxazole, pyrimethamine, ciproxin, ceftazidime, acetaminophen, codein, methadone, diclofenac, ranitidine, metoclopramide, domperidone, temazepam, brotizolam, fluoxetine and mianserine.

*Stability*

The stability of fluconazole was examined in eluent, at room temperature for 36 h, in plasma for 30 min at 60°C (thermal HIV-inactivation), in saliva in the saliva-collecting device for 48 h at room temperature and in saliva and plasma for 18 weeks at −30°C. The samples were analyzed immediately after the storage period.

## 3. Results and discussion

Liquid–liquid extraction followed by isocratic HPLC analysis with UV detection at 261 nm is an adequate technique for rapid and accurate determination of plasma and saliva levels of fluconazole. Several extraction fluids have been tested.

Extraction with ethyl acetate or 1-butanol resulted in 100% extraction recovery of fluconazole, but also with many interferences in the chromatograms. The use of the chloroform–1-propanol (4:1, v/v) mixture resulted in a reproducible, approximately 90% extraction recovery of both fluconazole and phenacetin from plasma, and in "clean" chromatograms. With this extraction mixture fluconazole was also reproducibly extracted from saliva with comparable efficacy. However, because of a too large variation of extraction recovery of phenacetin from saliva (recovery 84%, variation coefficient 21%, $n = 10$, at a saliva concentration of 4 $\mu$g/ml) the use of an internal standard was omitted in the analysis of fluconazole in saliva. The described method has been demonstrated to be accurate and reliable with and without the use of an internal standard (as shown in Tables 1 and 2). The described extraction technique, combined with measurement on a RP-18 analytical column, preceded by a guard column, provides a good resolution of fluconazole, internal standard and naturally present plasma or saliva components. Typical HPLC chromatograms of plasma samples are shown in Fig. 2.

Table 1
Accuracy, between-day and within-day precision for the analysis of fluconazole in plasma determined in triplicate on three different days

| Theoretical concentration (μg/ml) | Measured concentration (μg/ml) | Accuracy (%) | Precision (%) | | n |
|---|---|---|---|---|---|
| | | | Within-day | Between-day | |
| 0.5 | 0.4659 | 93.2 | 6.6 | 15.0 | 9 |
| 5.0 | 4.922 | 98.4 | 7.1 | 5.7 | 9 |
| 50.0 | 49.10 | 98.2 | 2.0 | 1.3 | 9 |

Chromatograms of saliva samples demonstrated less interferences (of endogenous substances) near the solvent front. For the validation of the method the report of the conference on "Analytical Methods Validation: Bioavailability, Bioequivalence and Pharmacokinetic Studies"–held in 1990–was used as a guideline [14].

Validation characteristics of the assay in terms of linearity, accuracy and precision at different concentrations of the analyte in both plasma and saliva have been tabulated (Tables 1 and 2). Because, for the analysis of fluconazole in saliva, the error-mean-square is greater than the day-mean-square, which results in a negative variance estimate, the between-day precision is footnoted with the explanation that no significant additional variation was observed as a result of performing the assay in different runs (Table 2). Correlation coefficients and the sum of the absolute %RCR values (Σ%RCR) were calculated over the concentration range of interest for four different response functions ($y$–$x$, $y$–$x$ with weighting factor $1/x$, $y$–$x$ with weighting factor $1/x^2$ and $\log y$–$\log x$). As the response function $y$–$x$ with weighting factor $1/x^2$, resulted in the lowest overall Σ%RCR value for both fluconazole analysis in plasma and saliva it was selected as the most appropriate model despite that the correlation coefficients were slightly lower, but $>0.99$, in comparison with the other functions. %RCR has shown to be more sensitive than correlation coefficients [13].

The lower limit of quantitation is 0.1 μg/ml in plasma (using 500-μl samples) and 1.0 μg/ml in saliva (using 250-μl samples). Because the method is sufficiently sensitive with UV detection at 261 nm it is not necessary to use detection at 210 nm, which carries the risk of increased interference by endogenous compounds.

None of the registered medications caused an interference with the assay. The stability experiments demonstrated that fluconazole is stable in the eluent at room temperature for at least 36 h, that the drug is stable in the saliva collecting device for at least 48 h at room temperature and that inactivation of HIV for 30 min at 60°C in

Table 2
Accuracy, between-day and within-day precision for the analysis of fluconazole in saliva determined in duplicate on three different days

| Theoretical concentration (μg/ml) | Measured concentration (μg/ml) | Accuracy (%) | Precision (%) | | n |
|---|---|---|---|---|---|
| | | | Within-day | Between-day | |
| 5.0 | 5.39 | 107.8 | 10.8 | [a] | 6 |
| 50.0 | 49.53 | 99.1 | 8.2 | [a] | 6 |
| 75.0 | 73.35 | 97.8 | 3.3 | [a] | 6 |

[a] No significant additional variation was observed as a result of performing the assay in different runs.

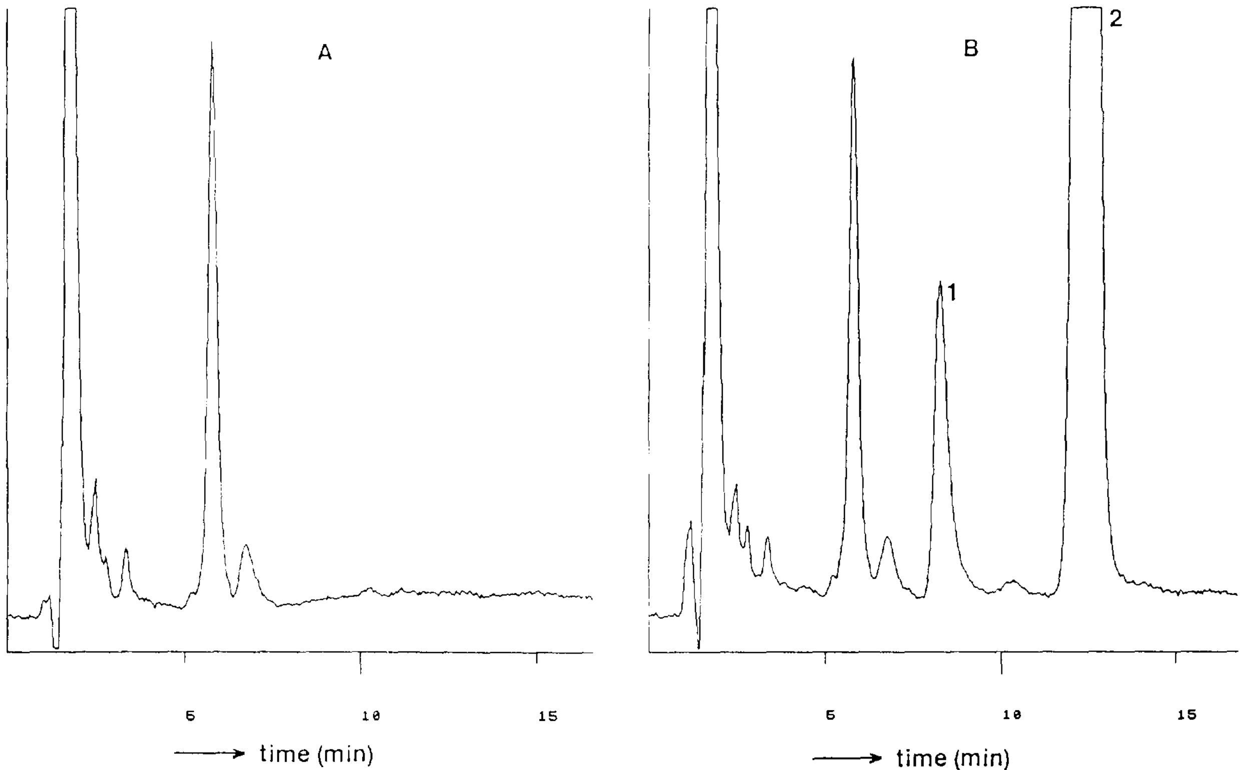


Fig. 2. HPLC of a blank patient plasma sample (A) and a patient plasma sample (fluconazole 3.65 μg/ml; peak 1) spiked with phenacetin (4 μg/ml; peak 2) (B).

plasma or storage at −30°C for 18 weeks in saliva or plasma had no influence on fluconazole concentrations.

The applicability of the assay for pharmacokinetic studies has been demonstrated in both patients and healthy volunteers. The fluconazole concentrations in plasma and saliva (in steady-state) after prolonged ingestion of 100 mg of fluconazole once a day by an HIV-infected patient with complaints of a "dry mouth" are shown in Fig. 3. It appears, in this case, that xerostomia has no negative influence on fluconazole saliva concentrations as the saliva concentrations are in the same order of magnitude as the plasma concentrations.

In conclusion, a simple, rapid and validated HPLC assay is described for the determination of fluconazole in saliva and plasma of HIV-infected patients. The sensitivity of the method is sufficient to monitor patients treated with fluconazole. The method is now used successfully in our hospital for pharmacokinetic studies with the drug.

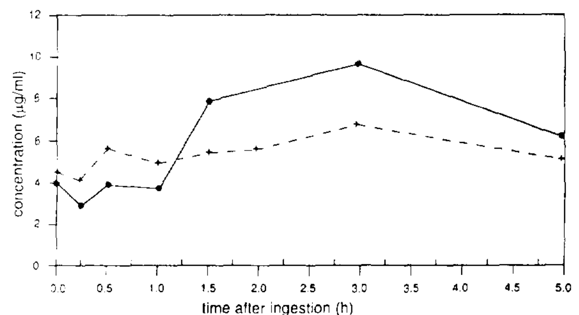


Fig. 3. Fluconazole saliva and plasma concentration–time curves (in steady state) after ingestion of 100 mg of fluconazole once a day in a patient with complaints of a dry mouth. (●) = saliva concentrations, ( + ) = plasma concentrations.

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