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Simultaneous determination of five systemic azoles in plasma by high-performance liquid chromatography with ultraviolet detection

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ABSTRACT

A simple, specific and automatable HPLC assay was developed for a simultaneous determination of systemic azoles (fluconazole, posaconazole, voriconazole, itraconazole and its metabolite hydroxyl-itraconazole, and ketoconazole) in plasma. The major advantage of this assay was sample preparation by a fully automatable solid phase extraction with Varian Plexa cartridges. C6-phenyl column was used for chromatographic separation, and UV detection was set at a wavelength of 260 nm. Linezolid was used as an internal standard. The assay was specific and linear over the concentration range of 0.05 to 40 μg/ml excepted for fluconazole which was between 0.05 and 100 μg/ml, and itraconazole between 0.1 and 40 μg/ml. Validation data for accuracy and precision for intra- and inter-day were good and satisfied FDA's guidance: CV between 0.24% and 11.66% and accuracy between 93.8% and 108.7% for all molecules. This assay was applied to therapeutic drug monitoring on patients hospitalized in intensive care and onco-hematologic units.

1. Introduction

The incidence of systemic fungal infections is rising with the increased frequency of causes of immunosuppression such as cancer chemotherapies, organ transplantation, or HIV infection. Several broad spectrum antifungals were recently developed; among them, systemic azoles are potent and selective inhibitors of 14α-demethylase (CYP51), which leads to depletion of ergosterol and accumulation of 14α-methyl-sterols, affecting the structure and function of the fungal membrane [1,2]. Recently marketed azoles as voriconazole and posaconazole, became a significant therapeutic class in invasive fungal infections.

These compounds are characterized by an important intra- and inter-individual variability of its pharmacokinetics parameters. Metabolized by cytochrome P450 enzymes (CYP3A4), they can generate many drug interactions [3]. These aspects suggest the necessity of performing a therapeutic drug monitoring of these antifungals in order to ensure an effective exposure of the drug, and to avoid risks of toxicity. Currently no method allows quantifying simultaneously systemic azoles. This study presents the development and validation of a simple and accurate method allowing simultaneous quantification of systemic azoles, and its application to therapeutic drug monitoring on patients hospitalized in intensive care and onco-hematologic units.

2. Experimental

2.1. Chemicals

Fluconazole and voriconazole were obtained from Pfizer (Paris, France); Ketoconazole, itraconazole and its metabolite hydroxyl-itraconazole were from Janssen-Cilag (Issy-les-Moulineaux, France); posaconazole was obtained from Schering-Plough (Levallois-Perret, France) and linezolid (internal standard) was from Pharmacia & Upjohn (Kalamazoo, USA). Acetonitrile and methanol HPLC quality were purchased from Scharlau (Barcelona, Spain). Di-natrium hydrogen phosphate Na_2HPO_4, concentrated orthophosphoric acid H3PO4 and ammoniac NH3 were from Prolabo (Nogent sur Marne, France).

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2.2. Equipment

Solid phase extraction was performed with a Gilson Aspec XLi automatic sample processor (Gilson Medical Electronics France, Villiers le Bel, France) using Bond Elute Plexa cartridges (Varian France, Courtabeuf). The HPLC system consisted of Agilent 1100 series (Agilent technologies, Weldbroon, Germany): a model G1311A quaternary pump, a model G1315B DAD UV detector and an Agilent Chemstation for LC systems.

2.3. Sample preparation

An automatic sample processor was used for the extraction (ASPEC Xli, Gilson). During the first step, the automate conditioned the Bond Elute Plexa cartridge by 1 ml of methanol and 1 ml of distilled water. 300 μl of sample was mixed with 500 μl of a 100 mM pH 2 phosphate buffer (KH_2PO_4), then with 250 μl of an internal standard solution (linezolid at 4 μg/ml) prepared in water. 1 ml of this mix was loaded; cartridge was washed with 1 ml of NH_4OH % and 1 ml of a mix of water and methanol 70/30 (v/v). Finally sample was eluted with 1 ml of methanol. The solvent was evaporated at 50 °C under a stream of nitrogen. The residue was reconstituted in 80 μl of a mix of water and methanol 50/50 (v/v), and 25 μl were injected into the chromatographic system.

2.4. Chromatography

The mobile phase consisted of 10 mM phosphate buffer adjusted to pH 7 with concentrated orthophosphoric acid (phase A), and mixed with acetonitrile (phase B); a gradient elution was performed with the two pumps: the gradient began with 75% and 25%, phase A and B, respectively, from 0 to 4 min, became 45% and 55%, phase A and B, respectively, from 5 to 11 min, then 20% and 80%, phase A and B, respectively, from 12 to 14 min and came back to original conditions between 14 and 19 min. The mobile phase was filtered through a 0.45 μm filter from Millipore (Saint Quentin en Yvelines, France), the flow rate was set at 1 ml/min. The analytical column was a Gemini C6-Phenyl (4.6 × 150 mm, 5.0 μm) from Phenomenex (Le Pecq, France). The sample injection volume was 25 μl. UV absorbance detection was set at 210 nm for fluconazole and 260 nm for the other azoles. The chromatographic run time was 19 min.

2.5. Preparation of calibration standards and quality controls

Stock solutions of 2 mg/ml of fluconazole, voriconazole, ketoconazole, posaconazole, hydroxy-itraconazole, itraconazole were prepared by appropriate dissolution of reference powders into methanol. Then, stock solutions were diluted into water containing 50% methanol to obtain a working solution of 200 μg/ml for fluconazole, 100 μg/ml for the others. This solution was diluted in free plasma to obtain a concentration range from 0.05 to 100 μg/ml of fluconazole, 0.1 to 40 μg/ml of itraconazole and 0.05 to 40 μg/ml of the others for calibration. For quality controls (QC), concentrations were different from those used for calibration and represented 0.15, 3.75, 15 and 75 μg/ml of fluconazole, 0.3, 1.5, 7.5 and 30 μg/ml of itraconazole and hydroxy-itraconazole and 0.15, 1.5, 7.5 and 30 μg/ml of other antifungals in plasma.

2.6. Sample treatments

The blood samples, received in free tubes BD Vacutainer systems (Becton-Dickinson, Le Pont-de-Claix, France), were centrifugated 10 min at 1200 × *g* (3000 tr/min) and the plasma was stored at −80 °C in cryovials until analysis. After being thawed, they were extracted by the automated solid phase process.

Different tubes with anticoagulants were also tested: EDTA and lithium heparinate, with no measurable effect on quantification of azoles in plasma.

2.7. Calibration and calculation procedure

Spiked matrix samples were prepared in a concentration range from 0.05 to 100 μg/ml depending on the antifungal; each calibration curve consisted of a blank sample and six calibrator concentrations. Daily calibration curves were constructed using the ratios of the observed peak areas of antifungals and internal standard (linezolid). Linear regression analysis of the calibration data was performed using the equation $y = mx + b$ where y was the peak area ratio, x the concentration of antifungal, m and b respectively the slope and intercept of the curve. Unknown concentrations were computed from the linear regression equation of the peak area ratio against concentration for the calibration curve.

2.8. Limit of detection and limit of quantitation

The limit of detection (LOD) was defined by the concentration with a signal-to-noise ratio of 3. The limit of quantitation (LOQ) was defined by the minimum amount that gave precise measurements (accuracy and precision both less than 20%) and was determined by injecting five times a number of spiked samples with decreasing concentration of the analyte.

2.9. Accuracy, precision and recovery

Accuracy was determined by the mean of the measured QC concentration relative to the theoretical value and was reported in percentage. The overall mean precision was defined by the coefficients of variation (CVs) as defined by the FDA guidelines [4]. The intra-day precision and accuracy for plasma QCs were calculated from three concentrations (0.15, 3.75, 15 and 75 μg/ml of fluconazole, 0.3, 1.5, 7.5 and 30 μg/ml of itraconazole and hydroxy-itraconazole and 0.15, 1.5, 7.5 and 30 μg/ml of other antifungals in plasma) analysed six times on the same day. For inter-day precision and accuracy, three QCs were analysed six times on four separated days ($n = 24$).

Recovery was evaluated by comparing the mean peak areas of the different QC samples post-extracted with those prepared by adding compound to post-extracted plasma at corresponding concentrations.

2.10. Specificity and selectivity

Six blank plasma samples from different healthy human sources were investigated for interference of endogenous matrix components. Specificity was assayed by adding molecules to blank human plasma: ropivacain (5 μg/ml), noradrenalin (5 μg/ml), epinephrine (2 μg/ml), norepinephrine (2 μg/ml), isoprenaline (2 μg/mL), ciclosporin (2 μg/ml), mycophenolic acid (10 μg/ml), sirolimus (2 μg/ml), tacrolimus (5 μg/ml), cyclophosphamide (2 μg/ml), fluorouracile (5 μg/ml), cytarabin (5 μg/ml), doxorubicin (5 μg/ml), gemcitabin (5 μg/ml), oxaliplatin (2 μg/ml), lopinavir (8 μg/ml), indinavir (5 μg/ml), atazanavir (3 μg/ml), fosamprenavir (4 μg/ml), nevirapin (5 μg/ml), tipranavir (10 μg/ml), efavirenz (10 μg/ml), tenofovir (4 μg/ml), ribavirin (3 μg/ml), acyclovir (2 μg/ml), caspofungin (2 μg/ml), ofloxacin, imipenem (10 μg/ml), piperacilline (10 μg/ml) tazobactam (0.5 μg/ml), gentamycin (2 μg/ml), tobramycin (5 μg/ml), amikacine (1.5 μg/ml),

rifampicin (5 μg/ml), ceftazidime (15 μg/ml), cloxacillin (5 μg/ml), ceftriaxone (6 μg/ml) and ciprofloxacin (2 μg/ml).

2.11. Effect dilution and memory effect

The effect dilution of plasma samples was validated with a high QC diluted to half, and analysed six times. Memory effect was tested by injecting three blank plasmas after the highest QC: percentage of disappearance of azoles was evaluated.

2.12. Stability

Stability of the six analytes was investigated in plasma. This stability was evaluated during sample collection, after short-term and long-term storage at −80 °C. Low, middle and high QC concentrations (0.15, 3.75 and 15 μg/ml of fluconazole, 0.3, 1.5 and 6 μg/ml of itraconazole and hydroxyl-itraconazole and 0.15, 1.5 and 6 μg/ml of other analytes) were prepared in six samples in plasma from a freshly made stock solution. These

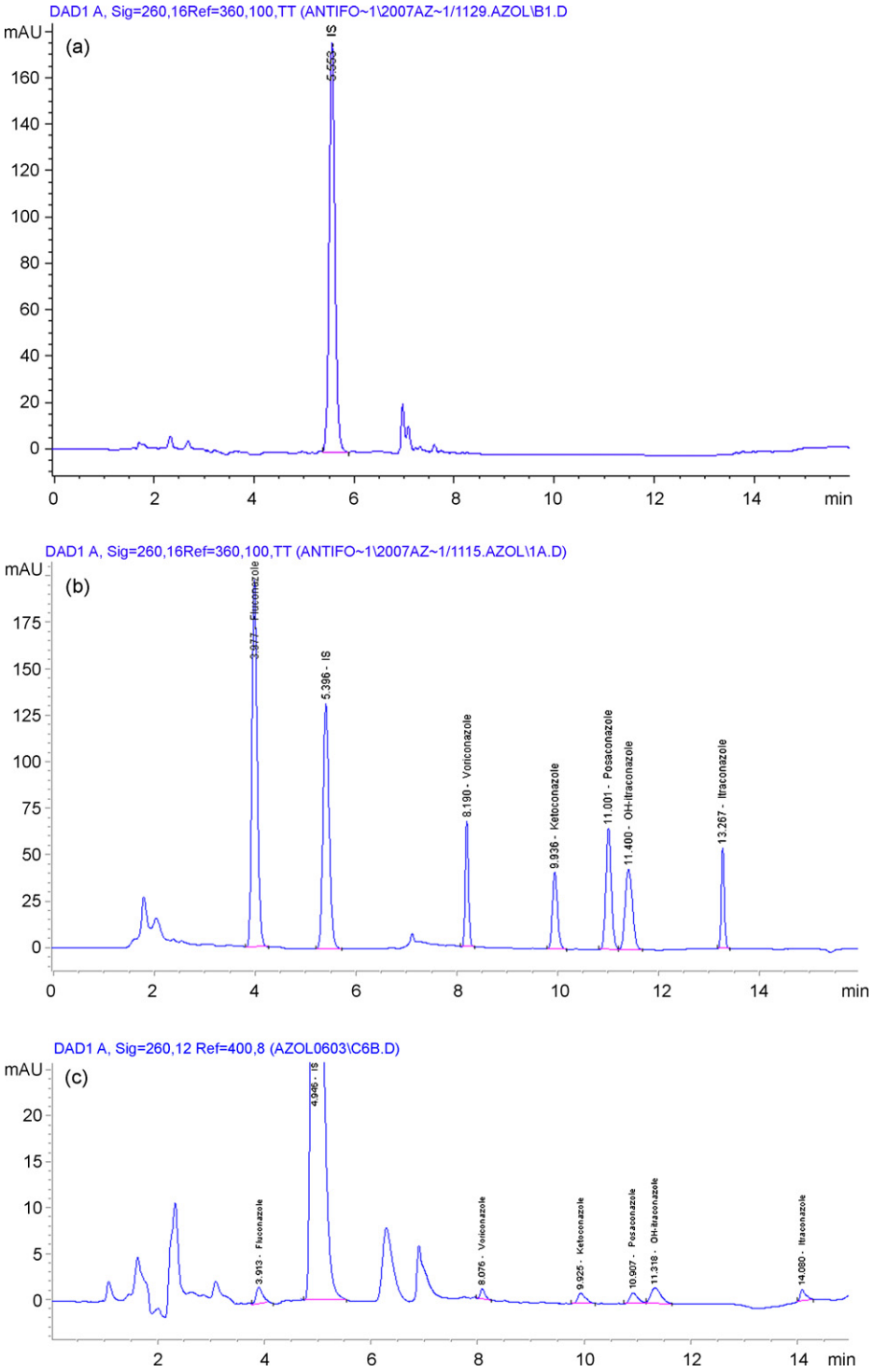

Fig. 1. Chromatograms used for validation analytical assay: chromatograms of blank human plasma sample (a), human plasma sample spiked with 15 μg/ml of fluconazole and 7.5 μg/ml of other antifungals (b), human plasma sample at LOQ level (spiked with 0.15 μg/ml of itraconazole and 0.05 μg/ml of other antifungals) (c).

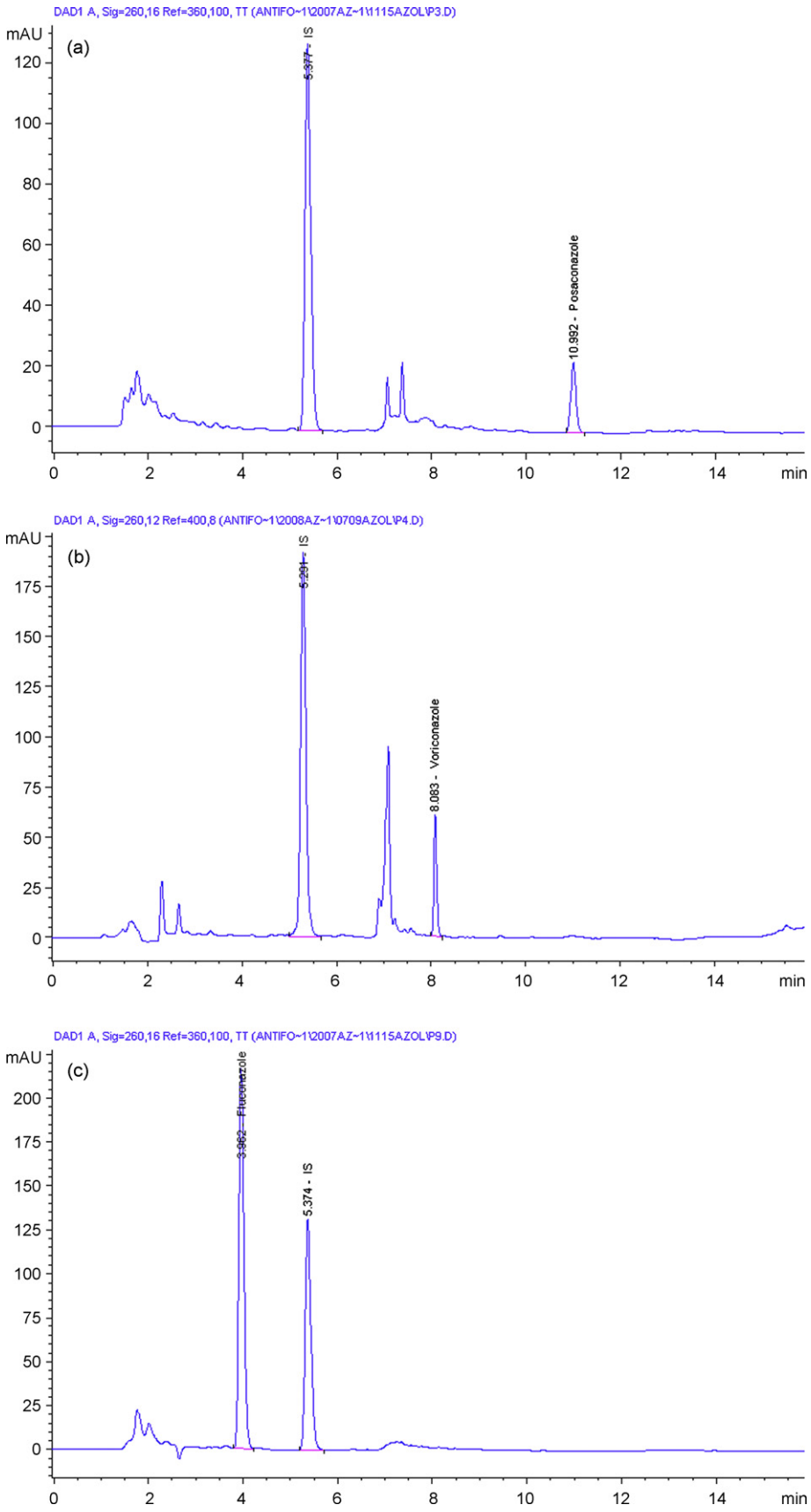

Fig. 2. Chromatograms used for determination of antifungals in patient's plasma: chromatogram of patient plasma sample treated with posaconazole with an observed concentration of 1.1 μg/ml (a); chromatogram of patient plasma sample treated with voriconazole with an observed concentration of 2.8 μg/ml (b); chromatogram of patient plasma sample treated with fluconazole with an observed concentration of 16.3 μg/ml (c).

QC were stocked at −80 °C. Antifungals' stability was determined by analysis of thawed QC concentrations after storage at room temperature for 24 h. Drug stability was also assessed from extracted QC concentrations ($n = 3$) stored in the sample tray of the autosampler for 24 h. For long-term stability, QC were stocked at −80 °C and were analysed at 3 and 6 months. The stability of stock solutions of antifungals and internal standard were assessed at room temperature for 24 h, and at −80 °C for 3 and 6 months.

Stability was validated if measured concentrations did not deviate from more than 10% from reference value obtained at T0.

Table 1
Intra-day and inter-day accuracy and precision.

	Theoretical concentration	Observed (mean ± SD)	Accuracy (%)	RSD	*n* (%)
Intra-day (μg/ml)					
Fluconazole	0.15	0.161 ± 0.011	107.3	6.83	6
	3.75	3.67 ± 0.021	97.9	0.57	6
	15	15.27 ± 0.253	101.8	1.66	6
	75	73.70 ± 0.661	98.3	0.90	6
Voriconazole	0.15	0.143 ± 0.012	95.3	8.39	6
	1.5	1.51 ± 0.004	100.5	0.24	6
	7.5	7.35 ± 0.107	98.0	1.46	6
	30	31.21 ± 0.599	104.0	1.92	6
Ketoconazole	0.15	0.162 ± 0.010	108.0	6.25	6
	1.5	1.45 ± 0.088	96.7	6.07	6
	7.5	7.46 ± 0.309	99.5	4.14	6
	30	28.97 ± 0.898	96.6	3.10	6
Posaconazole	0.15	0.147 ± 0.019	98.0	9.52	6
	1.5	1.54 ± 0.123	104.0	7.97	6
	7.5	7.25 ± 0.406	96.7	5.61	6
	30	30.98 ± 1.011	103.3	3.26	6
Itraconazole	0.30	0.285 ± 0.029	95.0	10.18	6
	1.5	1.57 ± 0.132	104.7	8.41	6
	7.5	7.40 ± 0.512	98.7	6.92	6
	30	28.59 ± 1.202	95.3	4.20	6
OH-itraconazole	0.30	0.324 ± 0.034	108.1	10.49	6
	1.5	1.54 ± 0.141	102.7	9.16	6
	7.5	7.59 ± 0.445	101.2	5.86	6
	30	29.01 ± 1.498	96.7	5.16	6
Inter-day (μg/ml)					
Fluconazole	0.15	0.163 ± 0.014	108.7	8.59	24
	3.75	3.72 ± 0.108	99.2	2.90	24
	15	15.22 ± 0.371	101.5	2.44	24
	75	72.10 ± 1.988	96.1	2.76	24
Voriconazole	0.15	0.157 ± 0.013	104.7	8.28	24
	1.5	1.53 ± 0.017	102.0	10.83	24
	7.5	7.46 ± 0.113	99.5	1.51	24
	30	32.07 ± 0.877	106.7	2.73	24
Ketoconazole	0.15	0.159 ± 0.012	106.1	7.55	24
	1.5	1.44 ± 0.091	96.2	6.32	24
	7.5	7.45 ± 0.275	99.3	3.69	24
	30	28.64 ± 1.008	95.5	3.52	24
Posaconazole	0.15	0.152 ± 0.009	101.3	5.92	24
	1.5	1.52 ± 0.098	101.3	6.45	24
	7.5	7.39 ± 0.413	98.5	1.84	24
	30	31.08 ± 1.117	103.6	3.59	24
Itraconazole	0.30	0.283 ± 0.033	94.3	11.66	24
	1.5	1.59 ± 0.147	106.0	9.25	24
	7.5	7.39 ± 0.571	98.5	7.73	24
	30	28.15 ± 1.812	93.8	6.43	24
OH-itraconazole	0.30	0.326 ± 0.036	108.7	11.04	24
	1.5	1.58 ± 0.149	105.3	9.43	24
	7.5	7.64 ± 0.515	101.9	6.74	24
	30	28.89 ± 2.005	96.3	6.94	24

3. Results

3.1. Chromatographic characteristics

Fig. 1a–c shows analytical chromatograms for blank plasma, quality control sample in plasma, and plasma spiked with azoles at LOQ level. Fig. 2a–c shows patient's chromatograms. The seven compounds were eluted in less than 15 min: the retention times are 4.0 min, 5.2 min, 8.1 min, 10 min, 11.2 min, 11.4 min and 14 min, respectively for fluconazole, linezolid, voriconazole, ketoconazole, posaconazole, hydroxy-itraconazole and itraconazole.

3.2. Calibration curve

The assay was linear from 0.05 to 100 μg/ml for fluconazole, from 0.1 to 40 μg/ml for itraconazole and hydroxy-itraconazole, and from 0.05 to 40 μg/ml for other analytes in plasma, and showed good correlation coefficients ($r^2 > 0.99$ for all analytes), with regression intercepts not statistically different from zero.

3.3. Limit of detection, limit of quantification

For itraconazole, the LOD and LOQ are determined in plasma as 0.05 and 0.15 μg/ml respectively; for the other antifungals, the LOD and LOQ are determined in plasma as 0.02 and 0.05 μg/ml respectively.

3.4. Specificity and selectivity

No endogenous substance did interfere in blank plasma with fluconazole, linezolid, voriconazole, ketoconazole, posaconazole, itraconazole and hydroxy-itraconazole. Potentially co-administered drugs tested had retention times that were different from analytes or were not detected.

3.5. Accuracy, precision and recovery

The intra-day variability calculated from three plasma QC samples six-fold injected on the same day was low, with precision

Table 2
Recovery determination.

	QC concentration	Recovery (%)
Fluconazole	0.15	88.3
	3.75	92.4
	15	91.6
	75	90.9
Voriconazole	0.15	87.6
	1.5	90.7
	7.5	91.1
	30	92.3
Ketoconazole	0.15	93.1
	1.5	97.6
	7.5	98.8
	30	96.8
Posaconazole	0.15	92.6
	1.5	91.9
	7.5	94.1
	30	93.5
Itraconazole	0.30	81.2
	1.5	82.9
	7.5	84.5
	30	83.7
OH-itraconazole	0.30	81.5
	1.5	85.1
	7.5	83.4
	30	86.2

Table 3
Pharmacokinetics data for voriconazole and posaconazole.

	N	Median	Min	Max	1st quartile	3rd quartile	IQR
Voriconazole C_{min}	24	0.830	0.250	2.780	0.520	1.165	0.645
Voriconazole C_{max}	12	3.575	1.100	8.800	2.515	4.650	2.135
Posaconazole	22	0.990	0.390	1.490	0.820	1.140	0.320

IQR: interquartile range.

ranging from 0.24% to 10.49% for CV and accuracy from 95.0% to 108.1%. The inter-day evaluation of plasma QC samples gave good results with precision from 1.51% to 11.66% for CV and accuracy from 93.8% to 108.7% (Table 1). The extraction mean recoveries of antifungals from QC samples on four separate days ranged from 81.2% to 98.8% (Table 2).

3.6. Effect dilution and memory effect

Quantification of half diluted QC gave reliable results: 104.3% for accuracy and 5.97% for CV. No memory effect was detected after injection of the highest QC: no azole was quantifiable in the three blanks.

3.7. Stability

Stock solutions of antifungals in methanol showed no perceptible degradation between solutions kept at room temperature for 24 h, at −20 °C for 3 and 6 months, and freshly prepared solutions. QC samples stored at −80 °C showed no significant degradation when analysed at 3 and 6 months: mean antifungal concentration ranged from 96.5% to 103.3% in plasma compared to freshly prepared QCs. At room temperature, thawed QC samples and extracted samples were stable, no significant loss was measured: mean concentrations ranged from 96.9% to 101.6% compared with references.

3.8. Therapeutic drug monitoring

The method was used to ensure optimisation of systemic antifungal treatments, particularly voriconazole, posaconazole and fluconazole on patients from intensive care and onco-hematology units. Data for posaconazole and voriconazole are shown in Table 3 and Fig. 3. Posaconazole exposures were similar between subjects, even with different doses (from 400 to 800 mg a day). Due to its long elimination half life, posaconazole presented flat concentration profiles at equilibrium. Patient 1, who received Noxafil® 200 mg twice a day, was monitored during 36 h and showed a very stable concentration (Fig. 3). With usual doses (400 to 800 mg bid), observed concentrations (Table 3) were comparable with those previously determined on infected patients, with a median maximal concentration at equilibrium close to 1 μg/ml [5]. For voriconazole with usual doses of 200 mg twice a day, a median C_{min} of 0.83 μg/ml and a median C_{max} of 3.57 μg/ml with a relatively high standard deviation were observed. For one patient, enzymatic induction by rifampicin decreased concentration of voriconazole to non-detectable values; switching this patient to posaconazole permitted to ensure a better exposition (about 0.5 μg/ml), but inferior to other patients treated with posaconazole, even increasing given doses to 200 mg × 4/day [6]. This method was adapted to manage with efficiency therapeutic drug monitoring on various patients treated with posaconazole and voriconazole, especially for drug–drug interactions.

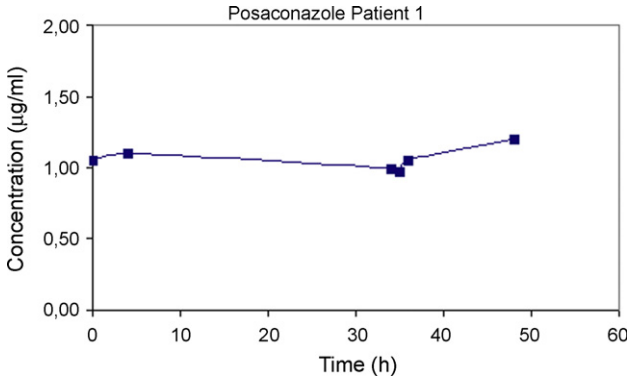

Fig. 3. Time profile of plasma concentrations of posaconazole in a patient treated with 200 mg bid.

4. Discussion and conclusion

A simple, specific and automatable HPLC assay was developed to quantify simultaneously systemic azoles in plasma using solid phase extraction and UV detection. LOD and LOQ were respectively 0.02 and 0.05 μg/ml for all drugs except itraconazole (0.05 and 0.15 μg/ml), which was better than previously reported HPLC assays [7–11]. This assay is the only one which allows quantifying simultaneously all systemic azoles. A single method permits saving analysis time, solvents and vials consumption. Our method uses equipment usually found in analytical laboratories, since with an HPLC-UV both sensitivity and specificity are sufficient. Chromatographic separation of posaconazole and OH-itraconazole for QCs and calibrations was challenging: with a C18 column these two analytes were co-eluted, consequently we chose a C6-phenyl column which permitted a good separation (resolution factor Rs > 1.5) thanks to enhanced aromatic interactions, especially based on π–π binding.

Another major advantage of this assay is the sample preparation by solid phase extraction. To obtain clean and reproducible chromatograms, an efficient sample extraction must be done. Previous assays used protein precipitation or liquid–liquid extraction [7–11]. Protein precipitation with acetonitrile or methanol is a simple procedure, with good recoveries but has some inconveniences: on the one hand, wide proportion of organic solvent could cause alteration of chromatographic parameters; on the other hand, this method is not specific and many endogenous and exogenous compounds may appear on chromatograms as interfering peaks. Liquid–liquid extraction is more specific than protein precipitation but is time and solvent consumer. Solid phase extraction has major advantages over previously described methods. First, extracted samples are cleaner, which preserves columns' lifetimes; secondly, specificity is very good, the major part of interfering compounds are eliminated thanks to selectivity of cartridge polymer and efficiency of washing steps. Our SPE method was easily automated on an Aspec XL system, which allowed a good reproducibility and saved operator times. We chose Varian Plexa® cartridges which polymeric sorbent provided the best results (recovery and specificity) for a moderated cost, compared with Oasis HLB® and Phenomenex StrataX®.

Finally this automatable method could easily be used in laboratories to manage efficiently therapeutic drug monitoring of systemic azoles.

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