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ABSTRACT
This paper reports the MEPS-HPLC-DAD method for the simultaneous determination of 12 azole drugs (bifonazole, butoconazole, clotrimazole, econazole, itraconazole, ketoconazole, miconazole, posaconazole, ravuconazole, terconazole, tioconazole and voriconazole) administered to treat different systemic and topical fungal infections, in biological samples. Azole drugs separation was performed in 36 min. The analytical method was validated in the ranges as follows: 0.02–5 μg mL⁻¹ for ravuconazole; 0.2–5 μg mL⁻¹ for terconazole; 0.05–5 μg mL⁻¹ for the other compounds. Human plasma and urine were used as biological samples during the analysis, while benzyl-4-hydroxybenzoate was used as an internal standard. The precision (RSD%) and trueness (Bias%) values fulfill with International Guidelines requirements. To the best of our knowledge, this is the first HPLC-DAD procedure coupled to MEPS, which provides the simultaneous analysis of 12 azole drugs, available in the market, in human plasma and urine. Moreover, the method was successfully applied for the quantitative determination of two model drugs (itraconazole and miconazole) after oral administration in real samples.

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MEPS-HPLC-DAD; method development; plasma and urine; sample preparation; azole antifungal drugs

Introduction
Imidazole drugs are organic water-soluble compounds showing a diazole aromatic heterocycle with non-adjacent nitrogen atoms, which has two equivalent tautomeric forms due to the presence of two nitrogen atoms in the backbone structure. The imidazole ring is widely present in various natural products, e.g. alkaloids, histamine, histidine, purine, which share the same 1,3-C₃N₂ basic ring with different substituents on the side chains. The imidazole also forms the key building block of different drugs possessing several biological activities, such as antibacterial, anticancer, antifungal and analgesic.

Triazoles represent another group of azole derivatives with a broader antifungal spectrum and safer profile than imidazoles. The triazole antifungal drugs include fluconazole, isavuconazole, itraconazole, posaconazole, pramiconazole, ravuconazole and voriconazole. Azoles (both imidazoles and triazoles) represent the active principle ingredient (API) of different drugs, which are topically or systemically administered as creams, shampoos, powders, tablets and capsules to treat fungal infections. Bifonazole, butoconazole, clotrimazole, econazole, ketoconazole, miconazole, tioconazole and terconazole are commonly formulated as creams and powders, whereas itraconazole, ketoconazole, posaconazole and voriconazole are formulated as tablets and capsules.
Pursuing our interest in the search and development of antifungal drugs\(^4\)–\(^{10}\), the aim of this project was the quantification ofazole derivatives, currently available in the market, in real samples through innovative microextraction techniques.

The analysis of the antifungal drugs in biological samples, e.g. plasma and urine, was usually carried out using high-performance liquid chromatography (HPLC) coupled to fluorimetric detector\(^11\)–\(^{13}\), UV/Vis\(^14\)–\(^{18}\), mass spectrometry (HPLC-MS)\(^19\), tandem mass spectrometry (HPLC-MS/MS)\(^20\)–\(^{22}\) and other highly hyphenated instrument configurations\(^23\). HPLC-diode array (DAD) or UV/Vis detectors are also used to quantify azoles in pharmaceutical products\(^24\)–\(^{26}\). A C\(_{18}\), C\(_8\) or C\(_6\)-phenyl stationary phase and acetonitrile, as mobile phase, are often used to set up a rugged and routine analytical method, which can be scaled up for the industrial quality control procedures.

Although several analytical methods were developed to quantitate azole drugs in pharmaceutical compounds, no HPLC-DAD method coupled to microextraction by packed sorbent (MEPS) technique is actually available to analyze simultaneously several azole derivatives in biological samples. Indeed, few works showed the separation of a limited number of triazoles, e.g. posaconazole and voriconazole\(^25\)–\(^{27}\), or fluconazole, itraconazole, voriconazole, posaconazole\(^28\), or fluconazole, itraconazole, voriconazole, posaconazole and ketoconazole\(^29\), or ketoconazole, tioconazole, econazole, miconazole and itraconazole\(^30\) from biological samples. Up to date, a proper method for the simultaneous quantification of all the derivatives in Figure 1, suitable for clinical applications, was not reported.

The clean-up and extraction procedure can decrease the recovery of compounds, which show different physical-chemical properties and limit the optimization of the method for multiple drugs determination. The liquid-liquid extraction (LLE) with organic solvents\(^15\)–\(^{27}\) and protein precipitation are widely used to extract drugs from biological samples\(^30\); unfortunately, they often show suitable performances on a limited number of drugs as demonstrated by the low quantitative recovery of bifonazole, butoconazole, ravuconazole and terconazole in the biological samples. To improve the extraction procedure, the solid phase extraction (SPE) procedure was proposed\(^30\). In the herein reported study, the SPE extraction was carried out testing different stationary phases, such as Oasis HLB, Strata-X and Sep-Pak. The SPE sorbents were conditioned according to the instructions from the manufacturer and to Gordien et al.’s protocol\(^28\). The SPE extraction increased drugs recovery (>60%). Unfortunately, for this procedure, a large amount of samples, buffers and organic solvents was required. Furthermore, the SPE procedure could be time-consuming and samples must be dried to reduce volume before HPLC analysis to achieve better LOQ values and to improve the signal-to-noise ratio. To overcome these drawbacks, MEPS microextraction technique was tested to optimize the recovery and the time of analysis, as previously observed in other works\(^31\)–\(^{33}\), and then applied to biological samples. In fact, this procedure increased the recovery of samples and decreased both the time of analysis and the use of organic solvents compared with SPE.

The obtained MEPS-HPLC-DAD protocol can provide several advantages for the concurrent extraction and analysis of azole derivatives in biological samples compared with other complex methods. Furthermore, the availability of a procedure for the determination of several analytes could be useful for clinical applications. The reported method showed comparable (or better) analytical performances to the others reported in the literature (see section Comparisons with existing methods).

### Materials and methods

#### Chemicals, solvents and devices

Bifonazole, butoconazole, clotrimazole, econazole, itraconazole, ketoconazole, miconazole, posaconazole, ravuconazole, terconazole, tioconazole, voriconazole and benzyl-4-hydroxybenzoate...
Plasma and urine samples preparation

About 170 μL of human blank plasma or urine were mixed with 20 μL of analyte working solutions and 10 μL of IS (1 μg mL⁻¹) and vortexed for 3 min (15% v:v of matrix modification for calibration and QC samples and 5% v:v of matrix modification for real samples).

Biological samples were diluted using trichloroacetic acid (TCA) (20 mg mL⁻¹) in 1:0.5 (v:v) ratio, centrifuged (12,000 x g for 10 min) and extracted by MEPS apparatus. The TCA treatment was carried out to denature the biological proteins, to hydrolyze the drug-protein binding and to reduce the sample density (widely used in biochemistry also for the precipitation of macromolecules, such as proteins, DNA and RNA). This acid was chosen to provide greater stability of the selected analytes in acidic conditions with respect to the reported harsh conditions (1.0 M perchloric acid). The supernatant was loaded in the MEPS, cleaned using water (200 μL), eluted and directly injected into the HPLC-DAD using methanol (20 μL). The analysis showed the presence of interference peaks, but a suitable recovery of drugs in terms of signal-to-noise ratio and peak areas compared with SPE treated samples.

The off-line extraction procedure was optimized as follows: (i) conditioning of sorbent with 3 x 150 μL of methanol and 3 x 150 μL of phosphate buffer (40 mM, pH 2.5); (ii) loading of plasma samples diluted 1:0.5 (v:v) with TCA (20 mg mL⁻¹) (8 x 150 μL) or urine samples diluted 1:0.5 (v:v) with TCA (20 mg mL⁻¹) (8 x 200 μL); (iii) washing with 1 x 200 μL of phosphate buffer (40 mM, pH 2.5) and methanol (90:10, v:v); (iv) elution of samples with 8 x 25 μL of methanol in a single vial by using an average flow rate of 10 μL s⁻¹, and then directly injected into the HPLC-DAD system. This optimized procedure is reported graphically in Figure 2.

Apparatus and chromatographic conditions

Analyses were performed using an HPLC Waters liquid chromatography (model 600 solvent pump, 2996 DAD, Waters Corporation, Milford, MA). Mobile phase was directly on-line degassed by using a Biotech 4CH DEGASI Compact (Biotech Inc., Onsala, Sweden).
Empower v0.2 Software (Waters Spa, Milford, MA) was used to collect and analyze the data.

Two different columns were used to optimize the chromatographic conditions: Luna C18 (250 × 4.6 mm, 5 μm particle size, Phenomenex, Torrance, CA) and Discovery C8 column (250 × 4.6 mm, 5 μm particle size, Supelco, Milan, Italy). The Luna C18 packing column connected to a Security Guard column (4.0 × 3.0 mm, 5 μm particle size; Phenomenex, Torrance, CA) was finally used to separate 12 azole drugs and IS. The columns were thermostatted at 25°C (± 1°C) using a JetStream2 Plus column oven during the analysis.

The maximum wavelength of 210 nm was used to obtain the best signal-to-noise ratio during HPLC-DAD analysis and the maximum values of LODs, LOQs, although these compounds can be detected at 250 nm, 243 nm and 220 nm. These secondary maximum wavelengths were used to further identify the azole drugs during the analysis (Supplementary material, Section S1 for analytes and IS UV/Vis spectra and Section S2 for system suitability test).

The HPLC system was optimized to set-up the better signal-to-noise ratio of drugs in a single chromatographic analysis, the best peak shape, an appropriate run-time and the better peak resolution. First, the analyses were performed in isocratic conditions using Luna C18 or Discovery C8 column as previously reported, in different mobile phases, made up from organic solvents and buffers with increasing ionic strength (15 mM, 30 mM, 40 mM and 50 mM)), in different temperature (25°C, 35°C and 40°C) and pH (2.5, 3.0 and 5.5) conditions. The flow rate was always kept at 1.0 mL min⁻¹ during the analysis.

Results showed that the retention times decreased by increasing the buffer ionic strength from 15 mM to 30 mM phosphate buffer (pH 2.5). The retention times of drugs were also reduced by increasing column temperature from 25°C to 40°C; unfortunately, a broader shape of peaks occurred, probably related to an increased mass transfer kinetics and a corresponding reduction in terms of resolution and peak symmetry. Similar results were obtained by increasing pH value from 2.5 to 5.5. Symmetric peaks were obtained both at 40 mM and 50 mM of phosphate buffer (pH 2.5). A suitable resolution was obtained using Luna C18 phosphate buffer (40 mM, pH 2.5) as solvent A, acetonitrile as solvent B, isocratic conditions (58:42, v:v) at 25°C.

This condition provided the simultaneous analysis of the 12 azole drugs, but the major drawback was the high-retention times of ravaconazole (61.8 min) and itraconazole (67.2 min). The long-retention times of these two drugs in addition to the run time analysis (>68 min) and the low signal-to-noise ratio hampered the isocratic condition preliminary performed. For these reasons, different gradient elution modes, at the same conditions of ionic strength, pH and temperature, were tested to decrease the run time and improve the signal-to-noise ratio. The optimized gradient elution was 0–16 min, 42% B; 16–18 min, from 42% to 70% B, linear; 18–21 min, from 70% to 80% B, linear; 21–28 min, 80% B; 28–36 min, 42% B. This gradient allowed separating simultaneously these drugs and IS without any overlapping and interferences during the analysis (Figure 3). By using the optimized separation conditions, a baseline resolution for different drugs and IS was carried out by 36 min and the resulting retention times are reported in Tables 1 and 2 (see also Supplementary material, Section S2 for System Suitability Test (SST) separation).

Summarizing, the optimized HPLC elution comprises mobile phase composed by phosphate buffer (40 mM, pH 2.5), as solvent A, and AcN, as solvent B, at a flow rate of 1.0 mL min⁻¹, and the following gradient elution: 0–16 min, 42% B; 16–18 min, from 42% to 70% B, linear; 18–21 min, from 70% to 80% B, linear; 21–28 min, 80% B; 28–36 min, 42% B.

Method validation

The validation of analytical method was carried out according to the International Guidelines in order to check LODs, LOQs, linearity, intra- and inter-day trueness and precision, selectivity, recovery, stability and parallelism test of different drugs in plasma and urine samples.

The LOQ of the method was defined as the concentration of the lowest standard on the calibration curve for which (a) the analyte peak was identifiable and discrete, (b) the analyte response was at least 10 times the response of the blank sample and (c) the analyte response was reproducible with a precision less than 20% and trueness better of 80–120%. The LOD was estimated at a signal-to-noise ratio of 3:1 by injecting a series of samples with known concentrations. Precision and trueness studies were carried out at the LOQ and at three QC concentration levels by injecting six different preparations of the analytes and calculating the RSD% and Bias% of the back-calculated concentrations. Calibration curves were calculated by analyzing six-times these eight non-zero concentration standards (0.02, 0.05, 0.10, 0.20, 0.50, 0.80, 2.00 and 5.00 μg mL⁻¹), for ravaconazole; 0.20, 0.30, 0.50, 0.80, 1.50, 2.00, 3.00 and 5.00 μg mL⁻¹ for terconazole; 0.05, 0.10, 0.20, 0.30, 0.50, 0.80, 2.00 and 5.00 μg mL⁻¹, for all other azole drugs) prepared in freshly spiked plasma (and urine).

Concentrations of the QC’s and unknown samples were calculated by interpolating their analyte peak area/Internal Standard area ratio on the calibration curve. Selectivity was tested by analyzing, under optimized chromatographic conditions, six blank plasma and six urine samples from different sources, and by comparing them with spiked ones at a concentration close to the LOQ.

Results and discussion

Optimization of MEPS extraction procedure

Up to date MEPS applications cover a large variety of organic compounds due to a great availability of sorbents (C2, C8, C18, C8 SCX, SCX, SAX, silica and molecularly imprinted polymers). For the herein considered analytes and for their chemical structures and a wide range of log Kow values (ranging from 1.0 for voriconazole to 6.70 of butoconazole), the C18 type could represent a valid starting point for extraction process optimization. The MEPS optimization was performed using the blank samples (plasma and urine) spiked with 0.15 μg mL⁻¹ of different azole drugs. Using these samples, the different parameters that could affect the extraction process were evaluated.

Effect of sample volume

During extraction procedure optimization, especially by using MEPS device, the sample volume covers an important role. In fact, when complex matrices were analyzed, matrix components could saturate the stationary phase, reducing the availability of functional groups that can retain the analyte. Additionally, the use of large sample volume could represent a limiting point for the method applicability, particularly when biological matrices are considered. For these reasons and based on previously validated MEPS procedures, the whole extraction process was tested and evaluated on a total sample volume of 150 μL for plasma and 200 μL for urine. Furthermore, in the MEPS procedure, the cycles...
number could also represent a critical point, due to the possible pre-concentration enhancement particularly during sample loading and sample elution. The effects of cycles number on process efficiency for the different analytes are reported in Figure 4.

**Figure 3.** Chromatograms obtained after the extraction and analysis of 12 azoles and benzyl-4-hydroxybenzoate (IS) at the wavelength of 210 nm, respectively (up, plasma sample and, down, urine sample: (a) blank sample, (b) blank sample spiked with 5 µg mL\(^{-1}\) of IS and (c) blank sample spiked with 5 µg mL\(^{-1}\) of IS and 4 µg mL\(^{-1}\) of different drugs). 20 µL of samples were injected during the analysis.

**Effect of type of washing solvent and organic solvent percentage**

To optimize the MEPS extraction, two different washing solvent systems were tested: (i) methanol and phosphate buffer (pH 2.5, 40 mM) at the ratios of 5:95 and 10:90 (v:v), (ii) methanol and
The back extracting solvent volume is another critical parameter for MEPS device lifetime and the number of analyses by a single MEPS nee-
dow extraction up to 7 min, plus further 10 min to centrifuge samples.

### Table 2. Mean linear calibration curve parameters performed by weighted-linear least-squares regression analysis of six independent eight non-zero concentration points in plasma samples.

| Analyte       | Linearity range (µg L\(^{-1}\)) | Slope\(^a\)       | Intercept\(^a\)    | Determination coefficient \((r^2)\) | Wavelength (nm) | Retention time (min) |
|---------------|----------------------------------|-------------------|-------------------|-----------------------------------|----------------|----------------------|
| Ketoconazole  | 0.05-5 (0.017 µg mL\(^{-1}\))\(^b\) | 0.07762 ±0.004085 | −0.00021 ±0.00019 | 0.9846                            | 5.49 ±0.12      |                      |
| Terconazole   | 0.2-5 (0.070 µg mL\(^{-1}\))\(^b\) | 0.1906 ±0.016364  | 0.01732 ±0.00207  | 0.9954                            | 6.63 ±0.14      |                      |
| Voriconazole  | 0.05-5 (0.017 µg mL\(^{-1}\))\(^b\) | 0.084313 ±0.00568 | 0.002999 ±0.00295 | 0.9917                            | 9.94 ±0.13      |                      |
| Bifonazole    | 0.05-5 (0.017 µg mL\(^{-1}\))\(^b\) | 0.373833 ±0.036575 | 0.01551 ±0.002246 | 0.9910                            | 11.23 ±0.30     |                      |
| Clotrimazole  | 0.05-5 (0.017 µg mL\(^{-1}\))\(^b\) | 0.1883 ±0.015952  | 0.01477 ±0.001127 | 0.9961                            | 12.75 ±0.45     |                      |
| Econazole     | 0.05-5 (0.017 µg mL\(^{-1}\))\(^b\) | 0.113067 ±0.014298 | 0.005134 ±0.000549 | 0.9908                            | 14.19 ±0.34     |                      |
| Etaconazole   | 0.05-5 (0.017 µg mL\(^{-1}\))\(^b\) | 0.129033 ±0.014347 | 0.003792 ±0.002456 | 0.9927                            | 17.84 ±0.45     |                      |
| Butaconazole  | 0.05-5 (0.017 µg mL\(^{-1}\))\(^b\) | 0.2452 ±0.022889  | 0.002835 ±0.004686 | 0.9925                            | 22.64 ±1.84     |                      |
| Miconazole    | 0.05-5 (0.017 µg mL\(^{-1}\))\(^b\) | 0.129033 ±0.020994 | 0.00083 ±0.003097 | 0.9922                            | 23.22 ±0.12     |                      |
| Posaconazole  | 0.05-5 (0.017 µg mL\(^{-1}\))\(^b\) | 0.1483 ±0.021392  | 0.00155 ±0.001385 | 0.9962                            | 24.01 ±0.09     |                      |
| Ruvaconazole  | 0.02-5 (0.007 µg mL\(^{-1}\))\(^b\) | 0.032007 ±0.002906 | 0.002364 ±0.000659 | 0.9902                            | 26.06 ±0.11     |                      |
| Itraconazole  | 0.05-5 (0.017 µg mL\(^{-1}\))\(^b\) | 0.059923 ±0.011036 | 0.015467 ±0.02012 | 0.9924                            | 28.31 ±0.10     |                      |

\(^a\)Values at 95% confidence intervals on the mean of six independent calibration curves.

\(^b\)The round brackets show the LOD values obtained from signal-to-noise ratio; the slope and intercept of calibration curve are expressed in µg mL\(^{-1}\).

Effect of number of draw–eject cycles

The instrument response, in terms of peak area, was optimized, by performing multiple draw–eject cycles because it improved the analytes' extraction. The use of MEPS also decreased the time of sample extraction up to 7 min, plus further 10 min to centrifuge samples.

Effect of type of eluting solvent and MEPS memory effects

The carry-over effect was evaluated using blank plasma (or urine) injected after the analysis of fortified biological samples at the upper limit of quantification (ULOQ, 5 µg mL\(^{-1}\)) and no memory effect of analytical procedure was obtained during the analysis.

Selectivity

The selectivity was performed using six blank biological samples collected from different healthy volunteers, according to ICH guidelines. The blank samples showed neither area values over 20% of LOQ areas at the analyte retention times nor over 5% of IS area at the drug retention time. This parameter was evaluated using blank biological samples (plasma and urine) extracted by MEPS device and analyzed with HPLC-DAD without any fortification (a), after fortification with IS (b) or drugs and IS (c) (Figure 3). Results demonstrated that the retention times of drugs were similar to those obtained for real samples and no interfering peaks were detected during the analysis at 210 nm (Figure 3). The peak before the retention time of ketoconazole was observed during the analysis; however, the peak intensity and its retention did not change in all chromatograms and did not affect the ketoconazole quantification both in plasma and urine samples.

Effect of type of cleaning solvent on device lifetime

The cleaning solvent adopted in the optimized MEPS procedure is pure methanol due to its high-elution capacity when the C\(_{18}\) stationary phase was used. The cleaning solvent choice was guided water with HCl (0.1 N) at the ratios of 5:95 and 10:90 (v:v). The obtained HPLC chromatograms showed that phosphate buffer (pH 2.5, 40 mM) and methanol (90:10, v:v) (1 × 200 µL) were the best performing solvent mixture, because it improved the analytes' extraction. The use of MEPS also decreased the time of sample extraction up to 7 min, plus further 10 min to centrifuge samples.

Effect of type of eluting solvent and MEPS memory effects

The back extracting solvent volume is another critical parameter that needs to be optimized in order to enhance the signal-to-noise ratio. We tested different methanol volumes, 50, 75, 100, 150, 200 and 300 µL, and observed a signal improvement until 150 µL before reaching a plateau. The methanol was used as an eluent to obtain the maximum recovery of drugs and increased the area of the peak for all compounds and IS.
also by the possible increase in device lifetime and reduction of carry-over phenomena. For these reasons, the MEPS device was washed using pure methanol (5 x 200 μL) before another extraction and no carry-over phenomena were observed. Furthermore, the device could be re-used up to 70–90-folds, without any loss of herein validated analytical performances, by using methanol.

Method validation

The matrix-matched calibration curves were calculated by analyzing six-times the non-zero concentration standards made in freshly spiked plasma or urine samples. The results were obtained by plotting the analyte/IS area ratio for each level of quantification versus the nominal concentration of each standard solution. The linearity of the method was evaluated by calculating the intercept, slope, determination coefficient and variation in the range 0.02–5 μg mL⁻¹ for ravuconazole, 0.2–5 μg mL⁻¹ for terconazole and 0.05–5 μg mL⁻¹ for other azole drugs (Tables 1 and 2). The different curves were linear over the range reported with coefficients (r²) > 0.9846 and weighing factor of (1/x²), which are according to International Guidelines36. The calibration curve parameters are reported in Table 1 for plasma sample and in Table 2 for urine sample. The LOQ values were 0.02 μg mL⁻¹ for ravuconazole, 0.2 μg mL⁻¹ for terconazole and 0.05 μg mL⁻¹ for other azole drugs. The LOD values were 0.007 μg mL⁻¹ for ravuconazole, 0.07 μg mL⁻¹ for terconazole and 0.017 μg mL⁻¹ for other azole drugs (Tables 1 and 2). The resulting values were calculated at a signal-to-noise ratio (3:1).

The within-assay precision (repeatability) of the same day was observed analyte area respect to a water-based sample spiked at the same concentration level and submitted to the same extraction procedure.

Comparisons with existing methods

The reported method showed several advantages to analyze simultaneously 12 azole drugs in terms of time-consuming, simplicity and routine instrumentation, analytical performances, applications in clinic and pharmaceutical fields. Additionally, it shows comparable performances with respect to FPSE-HPLC-PDA procedure reported in the literature40. This method also improved the detection and quantification limits for azole drugs in biological samples. Published data showed that few azoles (only up to five drugs) can be simultaneously quantified in biological samples using a single analytical procedure (Table 3). Indeed, different protocols used complex and highly hyphenated configurations23,41, which require time and solvent consuming for the samples extraction12,27,29 and to obtain low level of LOQs. Other procedures showed that few azoles can be simultaneously analyzed using methods previously reported; but only one protocol29 showed the simultaneous analysis of six azoles in cosmetic formulations with a total running time of 80 min and LOQ values similar to those obtained in this study with only 36 min of analysis (Table 3).

The LOQ values, which were carried out using MEPS-HPLC-DAD procedure, were similar to those obtained using more sensitive
| Sample                  | Analytes                  | Extraction Method | Instrument Setting up | Stationary Phase | Total Extraction Procedure Time (minutes) | Chromatographic Analysis Time (minutes) | LOQ (µg mL⁻¹) | Linear Dynamic Range (µg mL⁻¹) | Ref. |
|------------------------|---------------------------|-------------------|-----------------------|------------------|------------------------------------------|----------------------------------------|--------------|-------------------------------|------|
| Serum                  | Posaconazole              | Column switching  | HPLC-FLD              | C₁₈              | 3                                        | 17                                     | 0.1          | 0.1–5                         | 19   |
| Plasma/saliva          | Voriconazole              | LLE               | HPLC-FLD              | C₁₈              | 6                                        | 12                                     | 0.1          | 0.1–10                        | 12   |
| Plasma                 | Itraconazole/metabolite   | LLE               | HPLC-FLD              | C₁₈              | 25                                       | 30                                     | 0.005        | 0.005–0.5                     | 11   |
| Plasma                 | Itraconazole              | Protein precipitation | HPLC-UV/Vis         | C₁₈              | 10                                       | 0                                    | –            | –                            | 14   |
| Plasma                 | Voriconazole              | LLE               | HPLC-UV/Vis           | C₁₈              | 15, plus dryness                         | 20                                     | 0.1          | 0.1–20                        | 15   |
| Plasma                 | Posaconazole              | –                 | HPLC-PDA              | C₈               | –                                        | 65                                     | –            | 0.05–10                       | 16   |
| Plasma                 | Itraconazole              | Protein precipitation | HPLC-MS/MS           | C₁₈              | –                                        | –                                     | 0.001        | 0.001–0.5                     | 20   |
| Plasma                 | Voriconazole              | LLE               | HPLC-MS/MS            | C₁₈              | 12                                       | –                                     | 0.027        | –                            | 21   |
| Plasma                 | Posaconazole              | LLE               | HPLC-MS/MS            | C₁₈              | –                                        | –                                     | 0.001        | –                            | 22   |
| Interlaboratory Test   | Itraconazole              | –                 | –                     | –                | –                                        | –                                     | –            | –                            | 23   |
| Formulation Itraconazole | –                  | HPLC-UV/Vis         | Sb-Aq                 |                  | 10                                       | –                                     | –            | –                            | 24   |
| Formulation Voriconazole | –                  | HPLC-UV/Vis         | C₁₈                 |                  | 20                                       | –                                     | –            | –                            | 25   |
| Optimalic Voriconazole | Related impurities        | –                 | HPLC-UV/Vis           | C₁₈              | –                                        | –                                     | –            | –                            | 26   |
| Plasma                 | Voriconazole              | LLE               | HPLC-UV/Vis           | C₈               | 25, plus dryness                         | 20                                     | 0.2          | 0.2–10                        | 27   |
| Plasma                 | Itraconazole              | SPE               | HPLC-UV/Vis           | C₈-phenyl        | –                                        | 19                                     | 0.05         | 0.05–50                       | 30   |
| Plasma                 | Fluconazole               | LLE               | HPLC-UV/Vis           | C₁₈              | –                                        | –                                     | 0.001        | 0.001–50                     | –    |
| Plasma                 | Itraconazole/metabolite   | LLE               | –                     | –                | –                                        | –                                     | –            | –                            | –    |
| Cosmetic               | Ketoconazole              | Ultrasound and Liquid Extraction | HPLC-UV/Vis         | RP amide C₁₆    | 10, plus centrifugation                  | 80                                     | 0.05         | 0.05–100                     | 28   |
| Tioconazole            | Econazole                 | –                 | –                     | –                | –                                        | –                                     | –            | –                            | –    |
| Itraconazole           | Miconazole                | –                 | –                     | –                | –                                        | –                                     | –            | –                            | –    |
| Clotrimazole           | Itraconazole              | –                 | –                     | –                | –                                        | –                                     | –            | –                            | –    |
| Plasma                 | Itraconazole              | Protein precipitation | HPLC-MS             | C₁₈              | 10                                       | 13                                     | 0.03         | 0.03–8                       | 19   |
| Plasma                 | Voriconazole              | –                 | HPLC-MS               | C₁₈              | –                                        | 20                                     | 0.03         | 0.03–8                       | 25   |
| Plasma                 | Itraconazole              | Protein precipitation | HPLC-UV/Vis         | C₈-phenyl        | 5                                        | 17                                     | 0.05         | 0.05–10                      | 17   |
| Voriconazole           | Posaconazole              | LLE               | HPLC-DAD              | C₁₈              | 12, plus dryness                         | –                                     | 0.05         | 0.05–5                        | 18   |
| Plasma                 | Posaconazole              | Protein precipitation | HPLC-MS/MS          | C₁₈ and PFP      | 10                                       | 4                                     | 0.1          | 0.1–20                       | 42   |
| Plasma                 | Voriconazole              | LLE               | UPLC-UV/Vis           | BEH Phenyl       | 10, plus dryness                         | 6                                     | 0.05         | 0.05–10                      | 43   |
| Plasma                 | Voriconazole/metabolite   | LLE               | HPLC-MS/MS            | Kinetex F5 (PFP) | 34                                       | 7.6                                     | 0.005        | 0.005–2.5                     | 39   |
| Plasma                 | Itraconazole/methyl azole | LLE               | UHPLC-MS/MS           | Kinex F5 (PFP)   | 34                                       | 7.6                                     | 0.005        | 0.005–2.5                     | 39   |
| Plasma                 | Voriconazole              | LLE               | UPLC-UV/Vis           | BEH Phenyl       | 10, plus dryness                         | 6                                     | 0.05         | 0.05–10                      | 43   |
| Serum                  | Posaconazole              | Protein precipitation | UFLC-MS/MS          | C₁₈              | 6                                        | 4                                     | 0.1          | 0.1–10                       | 44   |
| Plasma/urine           | Ketoconazole              | MEPS              | HPLC-DAD              | C₁₈              | 7                                        | 36                                     | 0.05         | 0.05–5                       | Current paper |
| Terconazole            | Voriconazole              | –                 | –                     | –                | –                                        | –                                     | –            | –                            | –    |
| Bifonazole             | Clotrimazole              | –                 | –                     | –                | –                                        | –                                     | –            | –                            | –    |
| Tioconazole            | Econazole                 | –                 | –                     | –                | –                                        | –                                     | –            | –                            | –    |
| Butoconazole           | Miconazole                | –                 | –                     | –                | –                                        | –                                     | –            | –                            | –    |
| Ketoconazole           | Posaconazole              | –                 | –                     | –                | –                                        | –                                     | –            | –                            | –    |
| Itraconazole           | Itraconazole/metabolite   | –                 | –                     | –                | –                                        | –                                     | –            | –                            | –    |
| Tioconazole            | Econazole                 | –                 | –                     | –                | –                                        | –                                     | –            | –                            | –    |

PFP: pentafluorophenyl; RP: reversed phase; LLE: liquid–liquid extraction; SLE: solid-supported liquid extraction; plus dryness: required time for complete solvent evaporation is not reported; plus centrifugation: required time for sample centrifugation is not reported.
Table 4. Quantitative analysis of plasma or urine samples collected from healthy human volunteers after single oral dose of commercial capsules of itraconazole (100 mg) and commercial tablets of miconazole (500 mg). Plasma was collected 4 h after the oral administration of drugs, while urine samples were collected at different times.

| Matrix | Sample no. | Formulation | Dose (mg × 2/die) | Time (h) | Volume (mL) | Concentration (µg mL⁻¹) | Total amount (mg) | Concentration (µg mL⁻¹) | Total amount (mg) |
|--------|------------|-------------|-------------------|----------|-------------|------------------------|------------------|------------------------|------------------|
| Plasma | 1          | Capsules    | 100               | 4        | 4000        | 0.124 (±0.07)          | 0.546            | n.d.                   | n.d.             |
|        | 2          | Tablets     | 500               | 4        | 4000        | n.d.                   | n.d.             | 0.508 (±0.05)          | 2.235            |
| Urine  | 3          | Capsules    | 100               | 0        | 100         | n.d.                   | n.d.             | n.d.                   | n.d.             |
|        | 4          | Capsules    | 100               | 3        | 110         | n.d.                   | n.d.             | n.d.                   | n.d.             |
|        | 5          | Capsules    | 100               | 6        | 100         | n.d.                   | n.d.             | n.d.                   | n.d.             |
|        | 6          | Capsules    | 100               | 9        | 110         | n.d.                   | n.d.             | n.d.                   | n.d.             |
|        | 7          | Capsules    | 100               | 12       | 110         | n.d.                   | n.d.             | n.d.                   | n.d.             |
|        | 8          | Capsules    | 100               | 18       | 110         | n.d.                   | n.d.             | 0.402 (±0.09)          | 0.044            |
|        | 9          | Capsules    | 100               | 24       | 110         | n.d.                   | n.d.             | n.d.                   | n.d.             |
|        | 10         | Capsules    | 100               | 36       | 110         | n.d.                   | n.d.             | n.d.                   | n.d.             |
|        | 11         | Tablets     | 500               | 0        | 80          | n.d.                   | n.d.             | n.d.                   | n.d.             |
|        | 12         | Tablets     | 500               | 3        | 380         | n.d.                   | n.d.             | n.d.                   | n.d.             |
|        | 13         | Tablets     | 500               | 6        | 140         | n.d.                   | n.d.             | 0.136 (±0.08)          | 0.019            |
|        | 14         | Tablets     | 500               | 9        | 160         | n.d.                   | n.d.             | 0.229 (±0.04)          | 0.037            |
|        | 15         | Tablets     | 500               | 12       | 120         | n.d.                   | n.d.             | 0.102 (±0.07)          | 0.012            |
|        | 16         | Tablets     | 500               | 21       | 420         | n.d.                   | n.d.             | 0.098 (±0.06)          | 0.041            |
|        | 17         | Tablets     | 500               | 24       | 40          | n.d.                   | n.d.             | 0.048 (±0.03)          | 0.002            |
|        | 18         | Tablets     | 500               | 36       | 60          | n.d.                   | n.d.             | n.d.                   | n.d.             |

aTime between the last drug administration and sample collection.

bAccording to 19.

cIn round brackets are reported standard errors of the mean (SEM) obtained from three independent measures.

dUrine volumes collected from treated patients. n.d.: not detected.

and selective detectors, e.g. FLD 11 or very expensive MS and MS/MS 19,21,42. In many cases, the LOQs of MEPS-HPLC-DAD procedure showed better values (two-folds) than published data, which were obtained using few drugs 12,13,15,27. The use of benzyl-4-hydroxybenzoxazole as IS allowed monitoring the efficacy of the extraction and the analytical procedure.

As reported in Table 3, to analyze, a pharmaceutical formulation is necessary to validate a large linear dynamic range (e.g. from 0.05 to 100 µg mL⁻¹) 29, while this necessity is not mandatory when biological matrices are considered. In fact, the availability of a large dynamic range could help in reducing the number of over-range samples that need to be re-analyzed.

For azoles herein considered, the biological levels generally observed are close from low µg mL⁻¹ to medium ng mL⁻¹ range 1,7-19 for plasma, while for urine, it is necessary to validate low ng mL⁻¹ range due to the low percentage of unchanged analyte. For this reason, the use of large dynamic range is not necessary as well as the availability of a high sensitivity in biological matrix analyses. Additionally, the use of large range could affect the method accuracy (precision and trueness) at low concentration levels.

Application to real plasma and urine samples

The performances of analytical method were tested in plasma and urine samples collected from healthy volunteers after single oral administration of commercial capsules of itraconazole (100 mg) and tablets of miconazole (500 mg). Biological samples were extracted by MEPS device and quantified using HPLC-DAD according to the reported validated method. Table 4 reports the quantitative data obtained after real samples analyses (Supplementary material, Section S5 for real samples chromatograms).

A low concentration of itraconazole was obtained in plasma after 4 h. This value agreed with data previously reported by Hardin et al. 45, which reported an itraconazole concentration of 0.244 ± 0.090 µg mL⁻¹ at 4 h for a single dose of 2 × 100 mg/die per os. The low concentration of itraconazole in the urine suggested that this drug was excreted as non-active metabolites in urine (35%) and faeces (54%) 46.

The plasma concentration of miconazole partially agreed with previously reported data 46 for a single dose of 2 × 500 mg/die per os. In fact, a low amount of this analyte (<1%) was excreted unchanged in the urine; while ∼10–20% of this drug was metabolized before excretion 47. The residual unmodified drug (∼50%) could be eliminated through faeces 48.

Conclusions

The MEPS-HPLC-DAD procedure represents a suitable method to analyze simultaneously 12 azole drugs in plasma and urine samples collected from healthy volunteers. The analytical method was optimized using different columns and mobile phase compositions to have a short run time, which can separate several antifungal drugs and benzyl-4-hydroxybenzoxazole (IS). The best performance of the analytical method was carried out by using a Luna C18 column, a binary solvent system made from phosphate buffer (40 mM, pH 2.5) and AcN (58:42, v:v), and a flow rate of 1.0 mL min⁻¹. The gradient elution allows separating 12 azole drugs better than isocratic condition. The performance of MEPS-HPLC-DAD apparatus also depends on pH, ionic strength of the buffer, temperature of the column and solvents. Acid pH (2.5), 40 mM (ionic strength), 25 °C and acetonitrile (solvent elution) provided the best set-up to separate and analyze azole drugs by using a single run of 36 min.

The validation parameters showed that our experimental protocol can be used to detect and quantitate simultaneously bifonazole, butoconazole, clotrimazole, econazole, itraconazole, ketoconazole, miconazole, posaconazole, ravuconazole, terconazole, tioconazole, voriconazole in plasma and urine samples collected from healthy volunteers. Samples from human healthy volunteers after a single oral dose of commercial capsules of itraconazole (100 mg) and tablets of miconazole (500 mg) demonstrated that the selected azole drugs can be analyzed in real biological samples (plasma and urine) and showed analytical parameters similar to standard azole drugs, which are used to develop and validate the method.
No interferences were observed between drugs and biological samples during the validation of method and the analysis of real samples. The MEPS-HPLC-DAD is an easy and quick procedure, which can decrease significantly the variability and the time of the analysis. The MEPS-HPLC-DAD apparatus can provide several advantages for the simultaneous analysis of antifungal drugs in multiple therapy and pharmaceutical science. The MEPS-HPLC-DAD in off-line mode can represent an easy and quick analytical tool to separate and analyze several drugs in biological samples without using expensive apparatus and complex methods, which needed skilled operators.

Disclosure statement

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ORCID

Marcello Locatelli http://orcid.org/0000-0002-0840-825X

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