ORIGINAL ARTICLE

LIQUID CHROMATOGRAPHY TANDEM MASS SPECTROMETRY DETERMINATION OF FLUCONAZOLE LEVELS IN HUMAN PLASMA FOR BIOAVAILABILITY STUDIES

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Abstract

The increase of fungal infections requires active antimycotic agents, fluconazole being widely used. A high-throughput tandem mass spectrometry coupled liquid chromatography method (LC-MS/MS) was developed and validated for the determination of fluconazole. Separation was performed using a Zorbax SB-C18 column with mobile phase consisting of methanol and 0.1% formic acid in water (45:55, v/v) in isocratic elution, with a flow rate of 0.6 mL/min. Detection of fluconazole was performed in multiple reaction monitoring mode (m/z 219.9 from m/z 306.9) with positive electrospray ionization. Plasma samples were processed using a protein precipitation method with methanol. As internal standard, prednisone was used. Method validation was carried out for selectivity, linearity ($r > 0.9978$), intra-day and inter-day precision (CV < 7.3%) and accuracy (bias < 13.5%) over the concentration range of 105 - 5600 ng/mL plasma. Recovery for fluconazol was 100.6 - 108.2%. The analytical method developed is simple, sensitive and selective, requires small volumes of plasma and can be used in clinical and bioequivalence studies.

Rezumat

Creşterea numărului infecţiilor fungice necesită agenţi activi antimicotici, fluconazolul fiind utilizat pe scară largă. A fost dezvoltată şi validată o metodă de cromatografie cu lichide cu detecţie spectrometrică de masă în tandem (LC-MS/MS) pentru determinarea fluconazolului. Pentru separarea cromatografică s-a folosit o coloană Zorbax SB-C18, cu fază mobilă constituită din metanol şi 0,1% acid formic în apă (45:55, v/v) în eluţie izocratică şi debit de 0,6 mL/min . S-a efectuat monitorizarea în mod MRM a fluconazolului (m/z 219.9 din m/z 306.9) cu ionizare *electrospray* pozitivă pentru detecţie. Procesarea probelor de plasmă s-a efectuat folosind precipitarea proteinelor cu metanol. Prednisonul a fost utilizat ca standard intern. S-a efectuat validarea metodei prin determinarea selectivităţii, liniarităţii ($r > 0,9978$), preciziei (CV < 7,3%) şi acurateţei (bias < 13,5%) între zile şi în aceeaşi zi, în intervalul de concentraţii 105 - 5600 ng/mL plasmă. Regăsirea fluconazolului s-a încadrat în 100,6-108,2%. Metoda analitică dezvoltată este simplă, sensibilă, selectivă, necesită volume mici de plasmă şi poate fi utilizată pentru studii clinice şi de bioechivalenţă.

Keywords: fluconazole assay, LC/MS method, bioequivalence study

Introduction

An alarming increase of invasive and localized fungal infections has been observed, both community-acquired, occurring in patients with HIV infections, as well as nosocomial, which predominantly appear in immunocompromised patients [2, 18].

This is probably due to the increasingly use of immunosuppressive treatments or aggressive chemotherapy in neoplastic patients, an increase in the number of transplant patients, extensive use of wide-spectrum antibiotic therapy [12, 20].

Fluconazole, the first representative of a subclass of synthetic triazolic antifungal agents is a widely used antimycotic even in critical patients with conditions such as: meningitis caused by *Criptococcus neoformans* in case of immuno-depressed patients (HIV/AIDS), pulmonary coccidioidomycosis, systemic, urinary or oral infections, dermatomycosis (*Tinea pedis*, *Tinea corporis*, *Tinea cruris*, *Tinea versicolor*). It can be used for prophylactic purposes in patients with immunodeficiency syndrome to prevent recurrence of *Candida spp.* infections or meningitis caused by *Criptococcus neoformans*, especially in patients in advanced stages of immunodepression with a number of lymphocytes TCD4 < 200/ mm^3 [1, 3, 15]. It has fungicidal activity against most invasive fungi encountered in clinical practice (*Blastomyces dermatiditis*, *Coccidioides immitis*, *Histoplasma*

capsulatum) and dermatophytes, with a good activity against most *Candida albicans*, *Candida tropicalis*, *Candida parapsilosis*, *Candida lusitaniae*, *Aspergilus spp.*, *Criptococcus neoformans*, less susceptible on *Candida glabrata*. In infections caused by *Candida dubliniensis*, *Candida guilliermondii*, *Candida kefyr*, *Candida lusitaniae* safety and efficiency of fluconazole is not confirmed by clinical studies. *Candida krusei* is considered resistant to treatment with fluconazole, but some recently published studies show a reversal of resistance to fluconazole of this strain if ibuprofen is simultaneously used [17].

Fluconazole can be administered orally or parenteral, intravenously, pharmacokinetic properties being similar regardless of the administration route, having a bioavailability > 90%, a distribution volume of 0.8 L/kg, it penetrates the blood-brain barrier, achieving cerebrospinal fluid, sputum, vaginal discharge concentrations similar to plasmatic concentrations, and has a plasmatic half-life of approx. 30 hours (with intervals of 20 - 50 hours).

The main path for excretion of fluconazole is renal, approximately 60 - 80% of the administered dose being found unaltered in urine, only 11% being excreted as metabolites. Particular caution is advised when administering fluconazole in patients with chronic kidney disease, the dosage needing adjustments due to an inverse relation between plasmatic half-life and creatinine clearance: for a clearance of creatinine between 21-50 mL/min the dosage must be reduced to 50%, and if the clearance of creatinine is between 11-20 mL/min the dosage must be reduced to 25% [19].

Fluconazole is a highly selective inhibitor of cytochrome P450, izoenzymes 2C9 and 2C19 and a moderate inhibitor of cytochrome CYP3A4 fungic dependent of the enzyme lanosterol 14 alpha demethylase, this enzyme transforming lanosterol to ergosterol. Subsequent accumulation of 14 alpha-methyl-sterols is correlated with subsequent loss of ergosterol in the fungal cell membrane and can be responsible for the antifungal activities of fluconazole [9].

Particular attention should be granted to the risk of plasma concentrations of other drugs increasing when they are administered together with fluconazole, drugs that are metabolized using CYP2C9, CYP2C19 and CYP3A4 paths. Special clinical or potentially important actions were observed during treatment with fluconazole with some drugs such as: oral hypoglycaemic agents, oral anticoagulants (vitamin K antagonist - warfarin), phenytoin, cyclosporine, theophylline, voriconazole, tacrolimus, sirolimus, benzodiazepines, oral contraceptives, calcium channel blockers, nonsteroidal anti-inflammatory drugs and cyclooxygenase 2 (CoX2), inhibitors of HMG-CoA reductase.

The main goal of the present study was to develop and validate an analytical method for the quantification of fluconazole in human plasma samples with application in bioavailability studies and therapeutic drug monitoring.

Tandem mass spectrometry coupled liquid chromatography (LC-MS/MS) methods are increasingly used for the determination of drug plasma concentrations due to the precision, accuracy and sensitivity. Several pharmacokinetic studies have been carried out to determine fluconazole plasma levels some of which used LC-MS methods to quantify fluconazole in human plasma [5].

A number of various analytical methods are reported using high performance liquid chromatography (HPLC) for bio-analysis of fluconazole in biological matrices like plasma [10, 11, 13, 16] or urine [8]. The most widely used sample processing methods are LLE (liquid/liquid extraction) [5, 13, 16] and SPE (SPE - solid phase extraction) [8]. Both LLE and SPE however are techniques which may necessitate more time and may raise costs.

The main focus was in developing an analytical method suitable for bioavailability studies or therapeutic drug monitoring by fast and inexpensive sample preparation, short sample runtimes and full validation of performance parameters.

Materials and Methods

Reagents

High purity methanol of LC/MS grade was manufactured by VWR International (Radnor, USA), formic acid 98-100% of analytical grade was acquired from Lach-Ner (Czech Republic). Ultra-purified water was obtained from a Milli-Q Integral 3 Water purification system (Millipore - Milford, USA). Human blank plasma was supplied by the Regional Blood Transfusion Center Tirgu-Mures (Romania) and was obtained from the healthy volunteers.

Apparatus

A Thermo Fisher Scientific (Waltham, USA) Accela HPLC system consisting of an Accela 600 quaternary pump with inline degasser, a PAL HTS-xt auto-sampler, an Accela VIM 2303TX column compartment coupled with a Thermo Fisher Scientific TSQ Quantum Access Max detector was used for chromatographic separation. Other equipment used: two Centurion Scientific (Stoughton, United Kingdom) K3 centrifuges; Mettler-Toledo (Greifensee, Switzerland) XS204 analytical balance; a Velp Scientifica (Usmate Velate, Italy) Vortex mixer; an Elma Transsonic (Singen, Germany) ultrasonic bath; Mettler-Toledo Rainin series automatic pipettes.

Liquid chromatography tandem mass spectrometry conditions

Fluconazole and prednisone (internal standard - IS) were analytically separated at room temperature on

a Zorbax SB-C18 3.0 x 100 mm, 3.5 µm chromatographic column (Agilent Technologies) with a mobile phase consisting of methanol and 0.1% formic acid in water (45:55, v/v) in isocratic elution, with a flow rate of 0.6 mL/min. Multiple reaction monitoring (MRM) mode detection was carried out for fluconazole and prednisone, monitoring the transitions: 219.9 m/z derived from 306.9 m/z ion at a collision energy of 19 V for fluconazole and 341.2 m/z derived from 359.2 m/z ion at a collision energy of 12 V for prednisone. Positive ionization was performed with the heated electrospray ion source set to the following parameters: Spray voltage: 6000 V, vaporizer temperature: 378ºC, sheath gas pressure: 20 psi, Ion Sweep Gas Pressure: 0.0 psi, Aux Gas Pressure: 20 psi, capillary temperature: 205ºC, capillary offset: 35, Tube Lens Offset: 115, Skimmer Offset: -10, Collision pressure: 1.6 psi. The run-time was of 8.5 minutes.

Standard solutions

An IS solution with a concentration of 100 µg/mL prednisone in methanol was prepared. A stock solution of fluconazole in methanol (400 µg/mL) was diluted in the same solvent to 280 µg/mL and 42 µg/mL stock solutions. These two stock solutions were diluted with 0.1% formic acid in water to obtain the calibration working solutions and reference working solutions. The working solutions and IS solution were used for the preparation of plasma calibration standards with concentrations of 105 (lower limit of quantification - LLOQ), 210, 420, 840, 1680, 2800, 4200 and 5600 ng/mL, and quality control (QC) plasma samples with concentrations of 315 ng/mL (lower), 2380 ng/mL (medium) and 4900 ng/mL (higher). All plasma solutions were stored at -20ºC until analysed.

Pre-treatment of samples

Working solutions (50 µL) (after being mixed with 150 µL blank plasma) and plasma samples (200 µL) were spiked with IS (30 µL) and deproteinized with methanol (600 µL), mixed (2 min) and finally centrifuged (3 min at 10000 rpm). Volumes of 200 µL of the supernatant were mixed with 1000 µL of a 50:50 mixture of methanol and 0.1% aqueous formic acid solution in chromatographic vials and inserted into the auto-sampler. Sample volumes of 10 µL were injected into the HTS-HPLC-MS.

The study protocol was reviewed and approved by the National Ethics Committee and the National Agency for Medicines and Medical Devices and was performed according to standards determined in the Declaration of Helsinki. All subjects participating signed a written informed consent.

Method validation

Selectivity and specificity of the method were tested. For selectivity testing chromatograms of spiked plasma samples were compared to blank plasma sample chromatograms. Six sources of the appropriate blank matrix were used and were analysed and evaluated for interference against a sample spiked at the lower limit of quantification. Absence of interfering components was accepted because the response was 6.64% (less than 20%) of the lower limit of quantification for the analyte.

Carryover of the method was also investigated, by injecting a blank sample after the upper limit of quantification calibration standard (5600 ng/mL) on each of the five days of the validation process. The carryover in the blank sample injected after a high concentration did not exceed 20% of LLOQ for the analyte and 5% for the IS.

The instrument data system was used for automatic calculation of concentrations using the internal standard method. Calibration curves were designed from single calibration standards, they were linear, their equation was $y = ax + b$, using a weighting factor $1/y^2$, where **y** is the ratio of areas of the analyte and IS, **x** is the ratio between the analyte concentration and IS, **a**-slope, **b**-intercept.

Five samples (n = 5) of each quality control (QC) sample (lower - QCA, medium - QCB and, respectively, higher - QCC) plasma samples were analysed on the same day to determine intra-day precision (expressed as coefficient of variation, CV, %) and accuracy (expressed as relative difference between obtained and theoretical concentration, bias %). Five samples of each quality control sample (lower, medium and, respectively, higher QC), each one in a different run (one on each day of the five days of the validation) were also analysed in order to establish between-run accuracy and precision.

A study of the lower limit of quantification (LLOQ) was also carried out. Six different samples of LLOQ with a concentration of 105 ng/mL fluconazole were injected and analysed. Within and between run accuracy and precision for the LLOQ were calculated.

Stability of fluconazole was extensively studied. Long term stability, short term stability, post-preparative stability (auto-sampler stability), freeze-thaw stability, stock and IS solutions stability were all studied. Stock solution stability was proven for at least 48 hours at -20ºC. Long term stability of plasma samples at -20ºC was proven for at least the whole duration of the study (2 months). Short term stability at room temperature was proven for at least 4 hours, while post-preparative stability at auto-sampler temperature (room temperature) was proven for at least 24 hours. Freeze-thaw stability was proven for at least 4 cycles, each consisting of 22 hours of freezing at -20ºC and 2 hours of thawing at room temperature.

Dilution integrity was also studied by preparing a sample with the concentration above LLOQ (5600 ng/mL) having a concentration of 14000 ng/mL.

This sample was diluted with blank plasma to obtain samples with a concentration of 1400 ng/mL, a value within the calibration curve (dilution factor 10). Between-run, within-run accuracy and precision for these samples was calculated.

Matrix effect and recovery were investigated. For determination of the matrix effect and recovery, four QCA and four QCC samples were prepared in plasma, and one QCA and one QCC sample prepared with pure solvent (ultra-purified water). The recovery for fluconazole and prednisone was determined by calculating the ratio of the peak area in the presence of matrix, to the peak area in absence of matrix (in water). The IS normalized matrix factor (MF) was also calculated by dividing the MF of the analyte with the MF of the IS. The CV of the IS normalized MF was calculated from the four QCA and for the four QCC samples.

The effect of the anticoagulant on the analytical methods sensitivity and accuracy was studied by preparing samples using plasma with citrate-phosphate-dextrose solution with adenine (CPDA) as an anticoagulant and samples using plasma with ethylenediaminetetraacetic acid (EDTA) as an anti-coagulant. The two sets of samples were injected and compared.

Results and Discussion

The best spectrometric responses for fluconazole were obtained using heated electrospray positive ionization (HESI) mode in comparison to electro-spray negative ionization mode and atmospheric pressure chemical ionization (APCI). Fluconazole accepts a proton in the acidic mobile phase forming the molecular ion $[M+H]^+$ (*m/z* 306.9). Fluconazole was then fragmented to 219.9 m/z derived from 306.9 m/z ion using collision energy of 19 V, while the fragment with 341.2 m/z derived from 359.2 m/z ion at collision energy of 12 V was monitored for prednisone.

The analytical method developed was optimized to achieve good peak separation and symmetrical chromatographic peaks. The best chromatographic results were obtained with a Zorbax SB-C18 3.0 x 100 mm, 3.5 µm column and a mixture of methanol and 0.1% formic acid in water (45:55, v/v) mobile phase under isocratic conditions at room temperature with a flow rate of 0.6 mL/min. No interfering endogenous peaks were detected in human blank plasma at the retention times of fluconazole (1.6 min) and prednisone (7.3 min) (Figure 1).

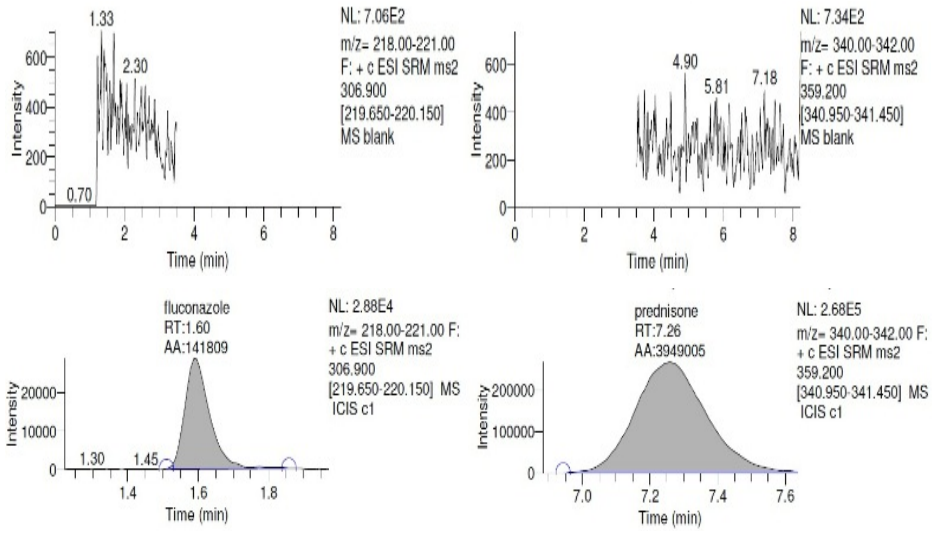

Figure 1.

Blank sample (upper figures) and LLOQ standard sample fluconazole and prednisone peaks (lower figures)

Linearity of the method was achieved, correlation coefficients of calibration curves were higher than 0.99 (R^2 between 0.9978 - 0.9987) throughout the concentration range studied (105 - 5600 ng/mL).

Within run and between run precision, accuracy and recovery results are shown in Table I and Table II. The LLOQ was set at 105 ng/mL with accuracy and precision below ± 20% (Table I and Table II).

Table I

Within-run accuracy, precision and recovery for fluconazole

$c_{nominal}$ (ng/mL)	Mean c_{found} (ng/mL (± SD))	Bias (%)	CV (%)	Mean recovery (% (± SD))
105 (LLOQ)	119.14 (4.95)	13.5	4.2	108.2 (6.8)
315 (QCA)	304.28 (22.12)	-3.4	7.3	101.1 (1.8)
2380 (QCB)	2407.18 (175.55)	1.1	7.3	102.3 (2.0)
4900 (QCC)	5144.80 (201.80)	5.0	3.9	106.2 (8.0)

Table II

Between run accuracy, precision and recovery for fluconazole

$c_{nominal}$ (ng/mL)	Mean c_{found} (ng/mL (± SD))	Bias (%)	CV (%)	Mean recovery (% (± SD))
105 (LLOQ)	115.00 (6.64)	9.5	5.8	105.3 (4.6)
315 (QCA)	306.13 (1.78)	-2.8	0.6	100.6 (1.4)
2380 (QCB)	2440.16 (31.42)	2.5	1.3	103.5 (2.4)
4900 (QCC)	5197.80 (186.76)	6.1	3.6	107.3 (7.6)

Various methods for the determination of fluconazole in biological matrices by HPLC or LC/MS, alone or simultaneously with other analytes [5, 8, 10, 11, 13, 16] have been described. Majcherczyk *et al.* developed and validated a single-step ultra-filtration method for extracting fluconazole from small amounts of plasma to determine its concentration by HPLC. Cellulose filters for ultra-filtration allows for a simple and reliable extraction, but it may not be available in some laboratories as it raises costs of analysis. The samples ultra-filtration and centrifugation took 60 min, but for clinical applications where hundreds or thousands of samples need to be processed this processing time may be too long [11].

Silvia Regina Cavani Jorge Santos *et al.* developed a high performance liquid chromatography (HPLC) method with IS to determine fluconazole. Sample extraction was carried out using liquid/liquid extraction by dichloromethane. This method can prove tedious when a large number of samples needs to be processed and requires use of reagents that can be toxic and raise costs [16].

Angela Eerkes *et al.* developed an LC-MS/MS method using the internal standard method. Sample transfer was performed using an automatic liquid handler and sample extraction was carried out using automated LLE, by usage of a 96-channel programmable liquid handling workstation. While the automation of sample transfer and LLE reduces sample processing time, automated samples transferring and/or automated LLE workstation are rarely available in most laboratories due to the very high costs [5].

Francois-Xavier Mathy *et al.* developed a method for the on-line determination of fluconazole in blood and dermal rat microdialysates by microbore high-performance liquid chromatography. The run time was 12 min for each sample, which is considerably longer compared to the run time of the newly developed method [12].

Sung-Su Kim *et al.* developed an HPLC method for determination of fluconazole plasma concentrations with applicability in bioequivalence studies. The concentration range for the method developed was between 0.05 and 10.00 µg/mL. Samples were processed using LLE with dichloromethane and total run time for each sample was 10 minutes. While this method was proven to have applicability in bioequivalence studies, the method developed by our laboratory had the advantage of easier and shorter sample processing times, shorter run times, better selectivity due to the use of tandem mass spectrometry. Using LC/MS also has the advantage of being a generally accepted technique by regulatory agencies due its specificity much greater than HPLC [10].

Fluconazole was also determined from other biological matrices. D Hermawan *et al.* developed and validated an HPLC method to determine fluconazole in urine after SPE using octadecyl-silane, with a percentage of recovery = 72.4%. The LLOQ was 1.28 µg/mL [8].

Our developed and validated method is simple and sensitive, uses small plasma sample volumes (200 µL) and requires a simple and inexpensive sample preparation methodology. A set of 20 samples (blanks, calibration standards, quality control samples) requires a preparation time of about 30 min. The large calibration range (105 – 5600 ng/mL) allows analysis of plasma samples in a wide concentration range.

Due to being subject of audit from the National Agency for Medicines and Medical Devices the method was fully validated in accordance with current international regulations [6, 7].

The method showed good linearity of the calibration curves, sensitivity (LLOQ of 105 ng/mL), was accurate and precise over the studied concentration range. Accuracy and precision limits provided by international guidelines [6, 7] were respected (Table I and Table II). The matrix effect and anti-coagulant effect studies show no major influence of the plasma or anticoagulant on the sensitivity, selectivity and accuracy of the method which means that the method has a high flexibility and can be used even in cases where a certain type of plasma and/or anticoagulant is not readily available.

The main goal of the present study was to develop and validate an LC-MS/MS analytical method for the fast, simple, sensitive and selective quantification of fluconazole in human plasma samples which can be used in therapeutic drug monitoring, bioavailability-bioequivalence studies or other pharmacokinetic studies (e.g. drug-drug interaction studies or pharmacokinetic approaches population). A typical plasma profile of fluconazole after a single administration of 150 mg doses of fluconazole to subjects is shown in Figure 2.

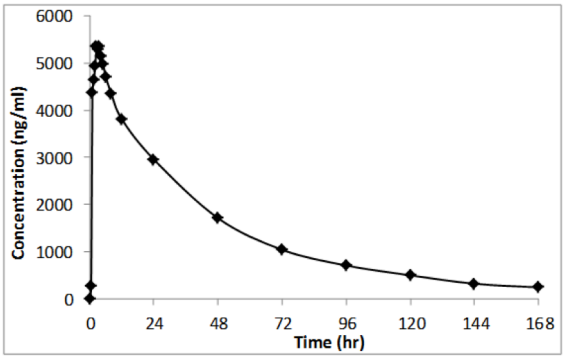

Figure 2.
Typical plasma profile of fluconazole administered by oral route, dose 150 mg, to a healthy volunteer

Conclusions

Our developed and validated LC-MS/MS method is simple, sensitive, selective and accurate. Compared to other methods reported in the scientific literature for the quantification of fluconazole in plasma [12-17] the method is simple, inexpensive and fast, features essential for high-throughput methods used in routine analysis. The method has wide applicability in pharmacokinetics, clinical level monitoring and clinical and bioequivalence studies.

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Conflict of interest

The main author is an employee of the sponsor of this study. The marketing authorization holder and manufacturer was the sponsor of this study.

References

1. Agudela C.A., Munoz C., Ramirez A., Response to therapy in patients with cryptococcosis and AIDS: Association with *in vitro* susceptibility to fluconazole. *Rev. Iberoam. Micol.*, 2014; PII: S1130-1406.
2. Bennett J.E., Introduction to mycoses. In Principles and practice of infectious diseases, Ed. VI, Phi, Churchill Livingstone 2005: 2935-2938.
3. Bicanic T., Harrison T.S., Cryptococcal meningitis. *Br. Med. Bull.*, 2005; 72: 99-118.
4. Burcea Dragomiroiu G.T.A., Cimpoieşu A., Ginghină O., Baloescu C., Bârcă M., Popa D.E., Ciobanu A.M., Anuţa V., The development and validation of a rapid HPLC method for determination of piroxicam. *Farmacia*, 2015; 63(1): 123-131.
5. Eerkes A., Shou W.Z., Naidong W., Liquid/liquid extraction using 96-well plate format in conjunction with hydrophilic interaction liquid chromatography/ tandem mass spectrometry method for the analysis of fluconazole in human plasma. *Journal of Pharmaceutical and Biomedical Analysis*, 2003; 31: 917-928.
6. Guidance for Industry. Bioanalytical Method Validation. U.S. Department of Health and Human Services. Food and Drug Administration. Center for Drug Evaluation and Research (CDER). Center for Veterinary Medicine (HESI M). September 2013, http://www.fda.gov/downloads/drugs/ guidance complianceregulatoryinformation/guidances/ucm368107.pdf
7. Guideline on bioanalytical method validation. European Medicines Agency. Committee for Medicinal Products for Human Use (CHMP). 275542/2014. http://www.ema.europa.eu/docs/en_GB/document_library/Scientific_guideline/2011/08/WC500109686.pdf
8. Hermawan D., Md Ali N.A., Wan Ibrahim W.A., Sanagi M.M., Analysis of Fluconazole in Human Urine Sample by High Performance Liquid Chromatography Method. *Journal of Physics: Conference Series*, 2013; 423: 012009.
9. http://www.ema.europa.eu/docs/ro_RO/document_library/Referrals_document/Diflucan_30/WC500121908.pdf
10. Kim S.S., Im H.T., Kang I.M., Lee H.S., Lee H.W., Cho S.H., Kim J.B., Lee K.T., An optimized analytical method of fluconazole in human plasma by high-performance liquid chromatography with ultraviolet detection and its application to a bioequivalence study. *J. of Chromatography B*, 2007; 852: 174-179.
11. Majcherczyk P.A., Moreillon P., Decosterd L.A., Sanglard D., Bille J., Glauser M.P., Marchetti O., Single-step extraction of fluconazole from plasma by ultra-filtration for the measurement of its free concentration by high performance liquid chromatography. *Journal of Pharmaceutical and Biomedical Analysis*, 2002; 28: 645-651.
12. Martin G.S., The epidemiology of sepsis in the United States from 1979 through 2000. *N. Engl. J. Med.*, 2003; 348: 1546-1554.
13. Mathy F.X., Vroman B., Ntivunwa D., De Winne A.J., Verbeeck R.K., Preat V., On-line determination of fluconazole in blood and dermal rat microdialysates by microbore high-performance liquid chromatography. *J. of Chromatography B*, 2003; 787: 323-331.
14. Negru J., Popa D.S., Vlase L., Iacob D., Achim M., Dorneanu V., High-throughput HPLC method for rapid quantification of ketoprofen in human plasma. *Farmacia*, 2015; 63(5): 770-775.
15. Oqbuagu O., Villanueva M., Extensive CNS Cryptococcal disease presenting as immune reconstitution syndrome in a patient with advanced HIV: report of case and review of management dilemmas and strategies. *Infect. Dis. Rep.*, 2014; 6(4): 5576.
16. Santos S.R.C.J., Campos E.V., Sanches C., Gomez D.S., Ferreira M.C., Fluconazole plasma concentration measurement by liquid chromatography for drug monitoring of burn patients. *Clinics*, 2010; 65(2): 237-243.
17. Sharma M., Biswas D., Kotwal A., Ibuprofen-Mediated Reversal of Fluconazole Resistance in Clinical Isolates of Candida. *J. Clin. Diagn. Res.*, 2015; 9(1): DC20-DC22.

18. Singh N., Trends in the epidemiology of opportunistic fungal infections: predisposing factors and the impact of antimicrobial use practice. *Clin. Infect. Dis.*, 2001; 33: 1692-1696.
19. Sinnollareddy M.G., Roberts M.S., Lipman J., Pharmacokinetics of fluconazole in critically ill patients with acute kidney injury receiving sustained low-efficiency diafiltration. *Int. J. Antimicrob. Agents*, 2015; 45(2): 192-195. Epub 2014 Oct 14.
20. Wisplinghoff H., Nosocomial bloodstream infections in US hospitals: analysis of 24.179 cases from a prospective nationwide surveillance study. *Clin. Infect. Dis.*, 2004; 39: 309-317.