



## VARIOUS QUANTITATIVE & QUALITATIVE METHODS FOR ESTIMATION OF ANTI FUNGAL DRUGS IN THEIR BIOLOGICAL SAMPLES –A REVIEW

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### ABSTRACT

This paper describes the various analytical methods reported for qualitative and quantitative estimation of various drugs in biological fluids, which are used in the treatment of fungal infection. Quantification of drugs in biological fluids is mandatory for the In vivo – In vitro correlation in order to maintain safety, efficacy and potency of the drug moiety. Quantification of active drug moiety in the presence of the metabolites and other biological products is typical task for the analyst, but inspire of costly extraction procedures, it's mandatory for therapeutic drug monitoring in order to assess the toxicity and to adjust the dose and it's frequency to maintain the therapeutic window.

**KEYWORDS:** Antifungal agents; Quantification; Biological fluids; LC-MS/MS.

### 1. INTRODUCTION

**Definition of fungal infections:** A fungal infection is characterized by nodular lesions first in the lungs and spreading to the nervous system like Candidiasis, Monilia disease, Moniliasis. An infection caused by fungi of the genus Monilia or Candida (especially Candida albicans).

#### Classification of Anti-Fungal Drugs

##### 1) Antibiotics:

- (i) **Polyenes:** Amphotericin B (AMB), Nystatin, Hamycin.
- (ii) **Echinocandins:** Caspofungin, Micafungin, Anidulafungin.
- (iii) **Heterocyclic benzofuran:** Griseofulvin.

##### 2) Antimetabolites: Flucytosine (5-FC).

##### 3) Azoles:

###### (i) Imidazoles:

(a) **Topical:** Clotrimazole, Econazole, Miconazole, Oxiconazole.

(b) **Systemic:** Ketoconazole.

(ii) **Triazoles (Systemic):** Fluconazole, Itraconazole, Voriconazole, Posaconazole.

##### 4) Allylamine: Terbinafine.

**5) Other Topical Agents:** Tolnaftate, Undecylenic acid, Benzoic acid, Quiniodochlor, Ciclopirox olamine, butenafine, Sodium thiosulphate.

#### Antifungal treatment strategies in high risk patients:

Different infections strategies for the treatment of invasive fungal in high risk patients with a focus on patients experiencing profound and prolonged Neutropenia, comprising those with acute myelogenous leukaemia or myelodysplastic syndrome during remission induction chemotherapy and on patients undergoing allogenic haematopoietic stem cell transplantation. Among those patients, invasive aspergillosis is the most frequently observed form of fungal infection, as opposed to high risk intensive care unit patients in whom an increased incidence of invasive candidiasis can be observed.

In both groups, initiation of early treatment has a profound impact on mortality rates, but adequate diagnostic tools are lacking. These circumstances have led to the parallel use of different treatment strategies, e.g. prophylaxis, empiric, pre-emptive and targeted treatment of invasive fungal infection. For empiric treatment of persistently febrile neutropenic patients, we opt for caspofungin as first and liposomal amphotericin B deoxycholate as second line choice. If the diagnosis of invasive aspergillosis can be established, voriconazole should be favoured over the alternative, liposomal amphotericin B. Patients with susceptible Candida spp. may be switched to fluconazole. Caspofungin or micafungin might be preferred to anidulafungin in the neutropenic patient.

**Table 1: Marketed formulations.**

DRUGS	BRAND NAME
Amphotericin B	Amphotericin B for injection, USP. Vial, 50mg.
Mycostatin, Nilstat	Nystatin Cream, USP. 15g.
Caspofungin, Caspofungin acetate	Cancidas Caspofungin, 70mg Powder for Injection; Caspoliv, 70mg Powder for Injection.
Micafungin	Mycamine, 50mg Vial.
Anidulafungin	Canidula (Anidulafungin for Injection, 100mg/ Vial)
Griseofulvin	S-Fulvin (Griseofulvin Tablets IP, 250mg)
Flucytosine	ANCOBON® (Flucytosine, 500mg Capsule)
Lotrimin, Mycelex.	Clotrimazole 1% Cream, USP (15g)
Econazole	Econazole Nitrate Cream 1% (15g)
Miconazole, Ornidazole	Candifem (Miconazole and Ornidazole Vaginal Cream, 30g)
Oxiconazole Nitrate Cream 1%	Oxistaj Cream, 1% (60mg)
Ketoconazole	Nizoral® Cream (Ketoconazole, 20g)
Fluconazole	FLUKA-150 (Fluconazole Tablets, 150mg); Diflucan (Fluconazole Capsules, 50mg)
Itraconazole	Sporanox-15D (Itraconazole Capsulas, 100mg)
Voriconazole	Vorzu and Voraze (Voriconazole Tablets, 200mg) Vorier (Voriconazole Injection, 200mg)
Posaconazole	Noxafil (Posaconazole Oral Suspension, 105ml)
Terbinafine	Daskil 1% Cream (Terbinafine HCL Cream 1%, 10g)
Tolnaftate	Tolnaftate 1% Antifungal Cream, 1oz.
Undecylenic acid, Berberine & Golden thread	Equate [Antifungal liquid Undecylenic acid, 1FL OZ (30ml)]
Benzoic Acid	Alpha Benzoic Acid Tablets, 1g.
Quiniodochlor	Entero Quinol (Quiniodochlor Tablets IP, 250mg)
Ciclopirox Olamine	Ciclopirox Olamine 15g Cream USP, 0.77%
Butenafine	Fintop Cream (Butenafine Hydrochloride Cream 1% w/w, 15g.
Sodium Thiosulphate	SAFE Dchlor T10 (Sodium Thiosulphate Tablets, 10mg)

## 2. Description

### 2.1: Amphotericin B

Su C, Yang H et al. reported LC-MS/MS method for quantification of Amphotericin B with the title "Bioanalysis of free and liposomal Amphotericin B in rat plasma using solid phase extraction and protein precipitation followed by LC-MS/MS" is described as follows: Amphotericin B (AMB) is a polyene macrolide antibiotic used for treating invasive fungal infections. Liposomal AMB (L-AMB) is a lipid dosage form which reduces the side effects and toxicity of the drug. The quantitation of free AMB (F-AMB) and L-AMB in vivo is important to monitor quality control of the liposomal formulation and to ensure its safety during clinical use. In this study, an original strategy was developed to separately determine F-AMB and L-AMB in rat plasma using LC-MS/MS. F-AMB was analyzed after separation by solid phase extraction, total AMB (T-AMB) was determined after protein precipitation and L-AMB was determined by difference. The method was fully validated. Calibration curves were linear in the ranges 0.7-120 µg/mL for T-AMB and 0.2-20 µg/mL for F-AMB. Accuracy and precision results were within acceptable variability limits, recoveries were consistent and reproducible, matrix effects were insignificant and analytes were stable under all the storage conditions

tested. The method was successfully applied to a pharmacokinetic study in rats administered a single intravenous 6 mg/kg dose of L-AMB. The method will allow further clinical studies of L-AMB and provide useful technical support for the assay of other liposomal drug formulations.<sup>[1]</sup>

### 2.2: Nystatin

Zhang D, Park JA et al. reported LC-MS/MS method for quantification of Nystatin with the title "Residual detection of buparvaquone, nystatin, and etomidate in animal-derived food products in a single chromatographic run using liquid chromatography-tandem mass spectrometry" is described as follows: A reliable and highly sensitive screening method based on liquid chromatography coupled with triple-quadrupole electrospray tandem mass spectrometry (LC-MS/MS) analysis has been developed for the detection and quantification of three veterinary drugs, including buparvaquone, nystatin, and etomidate impurity B CRS. The tested drugs were extracted from samples of porcine muscle, pasteurized whole milk, and eggs using 10mM ammonium formate in acetonitrile followed by liquid-liquid purification with n-hexane. Chromatographic separation was achieved on a Phenomenex Luna C18 analytical column using 0.1% formic acid in ultrapure

water (A) and acetonitrile (B) as mobile phases. All the matrix-matched calibration curves were linear ( $R^2 \geq 0.9756$ ) over the concentration levels of the drugs tested. Recovery at two spiking levels (equivalent to the limit of quantification (LOQ)=5ng/g and  $2 \times \text{LOQ}$ ) ranged from 72.88% to 92.59% with intra and inter-day precisions <17%, except for porcine muscle spiked with 5ng/g nystatin (RSD=25.15%). Samples collected from markets located in Seoul, Republic of Korea, tested negative for all the drugs analyzed. In summary, this method is suitable for screening and quantifying the selected drugs in a single chromatographic run and with high selectivity in animal-derived food products meant for human consumption.<sup>[2]</sup>

### 2.3 Caspofungin

Cheng X, Liu K et al. reported LC-MS/MS method for quantification of Caspofungin with the title "Development and validation of a liquid chromatography/tandem mass spectrometry method for determination of caspofungin in dried blood spots" is described as follows: A liquid chromatography/tandem mass spectrometry (LC/MS/MS) method for quantification of caspofungin in dried blood spots (DBS) was developed and validated. The DBS samples were prepared by spotting whole blood onto Whatman 903 filter paper, drying at room temperature and extracting with 50% methanol and further cleaned by protein precipitation with acetonitrile. Roxithromycin was selected as internal standard, and the separation of the analytes with endogenous ingredients was accomplished on a Hypersil GOLD aQ column with a mobile phase composed of 0.1% formic acid (v/v) and methanol in gradient mode. The detection of the analytes was performed on a triple quadrupole mass spectrometer in positive electrospray ionization mode, and the following selective reaction monitoring (SRM) transitions were monitored:  $m/z$  547.6  $\rightarrow$  538.7 and 837.4  $\rightarrow$  679.4 for quantification of caspofungin and the internal standard, respectively. The total analytical time was 8 min for each run. The calibration curve exhibited a good linearity over the range from 0.2 to 20  $\mu\text{g/mL}$  and the lower limit of quantification (LLOQ) was 0.2  $\mu\text{g/mL}$  for caspofungin in DBS. The recoveries of caspofungin ranged from 62.64% to 76.69%, and no obvious matrix effect was observed. The intra and inter-day precision and accuracy were within acceptable limits, and caspofungin in DBS was stable after storage at room temperature for 24 h and at  $-80^\circ\text{C}$  for 30 days. There was no evident effect of the hematocrit value on the analysis of caspofungin. The proposed method presents an alternative to the conventional venous sampling method, and was successfully utilized for pharmacokinetics study of caspofungin in ICU patient.<sup>[3]</sup>

### 2.4 MICAFUNGIN

Cangemi G, Barco S et al. reported LC-MS/MS method for quantification of Micafungin with the title "Quantification of micafungin in human plasma by liquid chromatography-tandem mass spectrometry" is described

as follows: Micafungin (MCF) is an antifungal agent of the echinocandin class approved in Europe both in adults and in children for the treatment of invasive candidiasis. Few analytical methods for therapeutic drug monitoring (TDM) of this drug have been described so far. In this paper, we describe a rapid and validated liquid chromatography-tandem mass spectrometry (LC-MS/MS) assay for the measurement of MCF in plasma. MCF was analyzed in 100- $\mu\text{L}$  plasma samples over a wide range of concentrations (0.1-20  $\mu\text{g/mL}$ ) by LC-MS/MS after protein precipitation. The suitability of the assay for TDM was evaluated by using plasma samples from pediatric patients who received MCF for the treatment of invasive candidiasis. The overall turnaround time for the assay was 20 min. The lower limit of quantification of the method was 0.1 ng/mL. No ion suppression due to matrix effects was found with different pre-analytical conditions, such as hemolysis, lipemia, and hyperuricemia. A simple and rapid LC-MS/MS method which provides high specificity, precision, and accuracy for quantification of MCF in plasma has been developed and validated.<sup>[4]</sup>

### 2.5 ANIDULAFUNGIN

Alebic-Kolbah T, Modesitt MS et al. reported LC-MS/MS method for quantification of Anidulafungin with the title "Anidulafungin—challenges in development and validation of an LC-MS/MS bioanalytical method validated for regulated clinical studies" is described as follows: Anidulafungin is a semi-synthetic echinocandin with antifungal activity, usually administered as an intravenous infusion. In order to determine the pharmacokinetics (PK) of anidulafungin in pediatric patients, a sensitive high performance liquid chromatography-tandem mass spectrometry (HPLC-MS/MS) bioanalytical method (M1) was developed and validated for quantification of anidulafungin in plasma. During analysis of incurred samples (samples collected from patients enrolled in a clinical study) an isobaric chromatographic interference was observed. The source of interference was identified as an anidulafungin opening form (D1) and its impact on the quantification of anidulafungin was investigated. It was found that accurately quantifying anidulafungin in incurred samples required chromatographic separation of the open-ring form from anidulafungin. The method was redeveloped to achieve the appropriate baseline separation and to avoid experimental conditions that favored opening the anidulafungin ring. The extraction of anidulafungin from plasma by protein precipitation remained unchanged, but the changes in chromatography warranted validation of a new method, M2, 2 years after M1 was validated. Incurred samples from three studies that were previously analyzed by M1 and were within confirmed long-term frozen stability were then reanalyzed by M2. Although the incurred sample reproducibility tests on those samples passed for each of the two methods, comparison of concentrations from the same samples obtained by M1 and M2 revealed that an over estimation of anidulafungin following the M1 method exceeded acceptance criteria.

The new HPLC-MS/MS method (M2) is applicable for quantification of anidulafungin within a nominal range 50-20,000 ng/mL and requires a 50  $\mu$ L human plasma aliquot. A linear, 1/concentration squared weighted, least-squares regression algorithm was used to generate the calibration curve and its parameters were used to quantitate the incurred samples. The inter-assay accuracy in heparin human plasma validation ranged from -4.33 to 0.0386 % and precision was  $\leq$ 7.32 %. The method M2 was validated for use in regulated bioanalysis and is presently used to quantitate anidulafungin in plasma samples from clinical studies.<sup>[5]</sup>

## 2.6GRISEOFULVIN

Mistri HN, Jangid AG et al. reported LC-MS/MS method for quantification of Griseofulvin with the title "Electrospray ionization LC-MS/MS validated method to quantify griseofulvin in human plasma and its application to bioequivalence study" is described as follows: A simple, sensitive and rapid liquid chromatography/tandem mass spectrometry (LC-MS/MS) method has been developed and validated to quantify griseofulvin in human plasma using propranolol hydrochloride as internal standard (IS). Samples were prepared using solid phase extraction and analysed without drying and reconstitution. The analytes were chromatographed on Hypersil, hypurity C18 reverse phase column under isocratic conditions using 0.05% formic acid in water:acetonitrile (30:70, v/v) as the mobile phase. Total chromatographic run time was 3.0 min. Quantitation was done on a triple quadrupole mass analyzer API-3000, equipped with turbo ion spray interface and operating in multiple reaction monitoring (MRM) mode to detect parent product ion transition for analyte and IS. The method was validated for sensitivity, matrix effect, accuracy and precision, linearity, recovery and stability studies. Linearity in plasma was observed over the concentration range 20-3000 ng/mL for griseofulvin. Lower limit of quantification (LLOQ) achieved was 20 ng/mL with precision (CV) less than 10% using 5  $\mu$ L injection volume. The absolute recovery of analyte (87.36%) and IS (98.91%) from spiked plasma samples was consistent and reproducible. Inter-batch and intra-batch coefficients of variation across four validation runs (LLOQ, LQC, MQC and HQC) was less than 7.5%. The accuracy determined at these levels was within  $\pm$ 4.2% in terms of relative error. The method was applied to a pilot bioequivalence study of 500 mg griseofulvin tablet in six healthy human subjects under fed condition.<sup>[6]</sup>

## 2.7 ECONAZOLE & CLOTRIMAZOLE

Yu, Ying; Zhang, Jing et al. reported LC-MS/MS method for simultaneous quantification of Econazole and Clotrimazole with the title "Development of a simple liquid chromatography-tandem mass spectrometry method for multiresidue determination of antifungal drugs in chicken tissues" is described as follows: A method involving LC coupled with MS/MS (LC/MS/MS) was designed for simultaneous

quantification of 10 antifungal drugs (voriconazole, griseofulvin, clotrimazole, bifonazole, econazole, ketoconazole, itraconazole, miconazole, terconazole, and fluconazole) in the liver and muscles of chickens. Homogenized tissue samples were extracted with acetonitrile and subsequently underwent freezing-delipidation. A Waters Acquity Ultra Performance LC BEH C18 column was used to separate the analytes, coupled with MS/MS using an electrospray ionization source. The accuracy of the method was confirmed with a mean recovery of 71-121%, and acceptable coefficients of variation (4-23%, n = 6). The detection capability of these compounds in two different matrixes was 0.50-2.82  $\mu$ g/kg. This method can be applied for the screening and confirmation of target antifungal drugs in chicken tissues.<sup>[7]</sup>

## 2.8MICONAZOLE

Du Y, Luo L et al. reported LC-MS/MS method for quantification of Miconazole with the title "Enantioselective separation and determination of miconazole in rat plasma by chiral LC-MS/MS: application in a stereoselective pharmacokinetic study" is described as follows: Miconazole has one chiral center, and consists of two enantiomers. In this study, a novel chiral liquid chromatography-tandem mass spectrometry method was developed for enantioselective separation and determination of miconazole in rat plasma. For the first time, the enantioselective pharmacokinetics of miconazole was investigated by the current method. Firstly, attempts were made to separate the enantiomers in reversed-phase mode with a mobile phase that was mass spectrometry compatible. Baseline separation was achieved on a Chiralpak IC column with a mobile phase composed of acetonitrile and aqueous ammonium hydrogen carbonate (5 mM; 80:20, v/v). Data were acquired in multiple reaction monitoring mode with positive electrospray ionization by triple-quadrupole mass spectrometry. Then, overall method validation regarding the linearity, accuracy, precision, extraction recovery, matrix effect, and stability of each enantiomer was performed, and acceptable results were obtained for all of these. Finally, the method developed was applied in an enantioselective pharmacokinetic study of miconazole enantiomers in rats after oral administration of racemic miconazole at doses of 5 and 10 mg/kg. The results demonstrated that (-)-(R)-miconazole had a higher concentration than (+)-(S)-miconazole in plasma, with a ratio of 1.3-1.7 for both doses. This is the first experimental evidence of enantioselective behavior of miconazole in vivo, and provides a reference for clinical practice and encourages further research into miconazole enantioselective metabolism and drug interactions. A stereoselective pharmacokinetic study of the miconazole enantiomers was investigated using a novel chiral liquid chromatography-tandem mass spectrometry method. Baseline separation was achieved on Chiralpak IC column, and Chiralcel OJ column was used to collect single enantiomer. A significant difference between the

two enantiomers was observed in view of the plasma concentration.<sup>[8]</sup>

### 2.9 KETOCONAZOLE

Wang K, Wu Y *et al.* reported LC-MS/MS method for quantification of Ketoconazole with the title “A highly sensitive LC-MS/MS method for determination of ketoconazole in human plasma: Application to a clinical study of the exposure to ketoconazole in patients after topical administration” is described as follows: A simple, rapid and highly sensitive LC-MS/MS method was developed and validated for the determination of ketoconazole in human plasma. Sample preparation was accomplished through a single step liquid-liquid extraction by ethyl acetate. The chromatography separation was carried out on a Hederia CN (150mm×2.1mm, 5µm) column with isocratic elution using acetonitrile and 10mM ammonium acetate containing 0.1% formic acid (45:55, v/v) as the mobile phase. The flow rate was 0.5mL/min. Detection was performed in the positive ion electrospray ionization mode using multiple reaction monitoring of the transitions of 531.2→489.3 and 286.1→217.1 for ketoconazole and Itraconazole (the internal standard), respectively. The method exhibited good linearity over the concentration range of 0.01-12ng/mL for ketoconazole. The intra and inter-batch precision and accuracy of ketoconazole were all within the acceptable criteria. The method was successfully applied to a clinical study of the exposure to ketoconazole in Chinese seborrheic dermatitis patients after topical administration of two ketoconazole formulations of foam and lotion, respectively. The study results showed that there was little systemic absorption of ketoconazole in patients for the two formulations, and the ketoconazole foam and lotion are safe therapeutic drugs for seborrheic dermatitis patients.<sup>[9]</sup>

### 2.10 FLUCONAZOLE

Astvad KMT, Meletiadis J *et al.* reported LC-MS/MS method for quantification of Fluconazole with the title “Fluconazole Pharmacokinetics in Galleria mellonella Larvae and Performance Evaluation of a Bioassay Compared to Liquid Chromatography-Tandem Mass Spectrometry for Hemolymph Specimens” is described as follows: The invertebrate model organism *Galleria mellonella* can be used to assess the efficacy of treatment of fungal infection. The fluconazole dose best mimicking human exposure during licensed dosing is unknown. We validated a bioassay. In conclusion, these *in vitro* data demonstrated that the formulation change is likely to have no significant impact on the bioperformance of 1% (w/w) butenafine hydrochloride cream or fluconazole detection in hemolymph and determined the fluconazole pharmacokinetics and pharmacodynamics in larval hemolymph in order to estimate a humanized dose for future experiments. A bioassay using 4-mm agar wells, 20 µl hemolymph, and the hypersusceptible *Candida albicans* DSY2621 was established and compared to a validated liquid chromatography-tandem mass

spectrometry (LC-MS-MS) method. *G. mellonella* larvae were injected with fluconazole (5, 10, and 20 mg/kg of larval weight), and hemolymph was harvested for 24 h for pharmacokinetics calculations. The exposure was compared to the human exposure during standard licensed dosing. The bioassay had a linear standard curve between 1 and 20 mg/liter. Accuracy and coefficients of variation (percent) values were below 10%. The Spearman coefficient between assays was 0.94. Fluconazole larval pharmacokinetics followed one-compartment linear kinetics, with the 24-h area under the hemolymph concentration-time curve (AUC<sub>24 h</sub>) being 93, 173, and 406 mg · h/liter for the three doses compared to 400 mg · h/liter in humans under licensed treatment. In conclusion, a bioassay was validated for fluconazole determination in hemolymph. The pharmacokinetics was linear. An exposure comparable to the human exposure during standard licensed dosing was obtained with 20 mg/kg.<sup>[10]</sup>

### 2.11 ITRACONAZOLE

Liang X, Van Parys M *et al.* reported LC-MS/MS method for quantification of Itraconazole with the title “Simultaneous determination of itraconazole, hydroxy itraconazole, keto itraconazole and N-desalkyl itraconazole concentration in human plasma using liquid chromatography with tandem mass spectrometry” is described as follows: A high-performance liquid chromatography tandem mass spectrometry (LC-MS/MS) assay was developed and validated for simultaneous determination of itraconazole (ITZ), hydroxy-itraconazole (OH-ITZ), keto-itraconazole (keto-ITZ) and N-desalkyl itraconazole (ND-ITZ) concentration in human plasma. One hundred and fifty microliters of human plasma were extracted using a solid-supported liquid extraction (SLE) method and the final extracts were analyzed using reverse-phase chromatography and positive electrospray ionization mass spectrometry. The standard curve range is 5-2500 ng/mL for ITZ and OH-ITZ and 0.4-200 ng/mL for keto-ITZ and ND-ITZ. The curve was fitted to a 1/x(2) weighted linear regression model for all analytes. The precision and accuracy of the LC-MS/MS assay based on the five analytical quality control (QC) levels were well within the acceptance criteria from both FDA and EMA guidance for bioanalytical method validation. Average extraction recovery was 97.4% for ITZ, 112.9% for OH-ITZ, 103.4% for keto-ITZ, and 102.3% for ND-ITZ across their respective curve range. Matrix factor was close to 1.0 at both high and low QC levels of all 4 analytes, which indicates minimal ion suppression or enhancement in our validated assay. Itraconazole and all three metabolites are stable in human plasma for 145 days stored at -70 °C freezers. The validated assay was successfully applied to a clinical study, which has a drug-drug interaction (DDI) arm using ITZ as a cytochrome P450, family 3, subfamily A (CYP3A) inhibitor.<sup>[11]</sup>

### 2.12 VORICONAZOLE

Li J, Ma J et al. reported LC-MS/MS method for quantification of Voriconazole with the title "A rapid ultra-performance LC-MS/MS assay for determination of serum unbound fraction of voriconazole in cancer patients" is described as follows: Voriconazole (VOR), an antifungal agent, is clinically monitored to guide therapeutic dosing and avoid toxicity. It is believed that measurement of serum unbound VOR provides more accurate information, especially in hypoalbuminemia patients. Developed and validated an accurate, simple and fast test with ultrafiltration and ultra-performance liquid chromatography (UPLC)-tandem mass spectrometry (MS/MS) to measure unbound VOR in human serum. The Agilent UPLC system coupled with a SCIEX QTRAP4000 MS with a positive ionization mode was developed and validated for VOR analysis. A good linearity was demonstrated from 0.02 to 2.5 µg/ml for unbound VOR ( $r^2=0.9969$ ). The within-run and between-run accuracy and precision was <5% and <6%. The levels of total VOR were well correlated with reference laboratory results. Serum unbound VOR levels were correlated with the total VOR levels ( $r=0.78$ ,  $p<0.0001$ ). There was a reverse correlation between unbound VOR fractions and plasma albumin levels ( $p<0.05$ ). In hypoalbuminemia patients, the unbound VOR levels were increased to a higher degree than total VOR. This assay is suitable for monitoring both unbound and bound VOR in cancer patients especially in those with hypoalbuminemia in clinical laboratories. Measurement of unbound VOR offers a better approach in prediction of VOR toxicity.<sup>[12]</sup>

### 2.13 POSACONAZOLE

Reddy TM, Tama CI et al. reported LC-MS/MS method for quantification of Posaconazole with the title "A dried blood spots technique based LC-MS/MS method for the analysis of posaconazole in human whole blood samples" is described as follows: A rugged and robust liquid chromatographic tandem mass spectrometric (LC-MS/MS) method utilizing dried blood spots (DBS) was developed and validated for the analysis of posaconazole in human whole blood. Posaconazole fortified blood samples were spotted (15 µL) onto Ahlstrom Alh-226 DBS cards and dried for at least 2h. Punched spots were then extracted by using a mixture of acetonitrile and water containing stable labeled internal standard (IS). Posaconazole and its IS were separated from endogenous matrix components on a Kinetex™ C18 column under gradient conditions with a mobile phase A consisting of 0.1% formic acid and a mobile phase B consisting of 0.1% formic acid in acetonitrile/methanol (70/30, v/v). The analyte and IS were detected using a Sciex API 4000 triple quadrupole LC-MS/MS system equipped with a TurbolonSpray™ source operated in the positive ion mode. The assay was linear over the concentration range of 5-5000 ng/mL. The inter-run accuracy and precision of the assay were -1.8% to 0.8% and 4.0% to 10.4%, respectively. Additional assessments unique to DBS were investigated including sample spot homogeneity,

spot volume, and hematocrit. Blood spot homogeneity was maintained and accurate and precise quantitation results were obtained when using a blood spot volume of between 15 and 35 µL. Human blood samples with hematocrit values ranging between 25% and 41% gave acceptable quantitation results. The validation results indicate that the method is accurate, precise, sensitive, selective and reproducible.<sup>[13]</sup>

### 2.14 TERBINAFINE

Gurule S, Khuroo A et al. reported LC-MS/MS method for quantification of Terbinafine with the title "Rational design for variability minimization in bioanalytical method validation: illustration with LC-MS/MS assay method for terbinafine estimation in human plasma" is described as follows: Terbinafine, a widely used antifungal drug, is a challenging molecule for quantitative bioanalysis due to certain factors contributing assay variability. Despite previous attempts at human plasma determination of terbinafine, exhaustive stability of the drug or an internal standard was lacking. Internal standard stability with negligible variation throughout the analysis is an indicator of a reliable bioanalytical method as the majority of LC-MS/MS assays are based on analyte/IS response ratios for quantitation. A newly developed high-throughput simple LC-MS/MS method is described for human plasma determination of terbinafine using naftifine internal standard and eluting all compounds within 2 min. A solid-phase extraction of terbinafine achieving mean recovery of 84.3% (CV < 4%) without compromising sensitivity (limit of quantitation 5.11 ng/mL) or linearity (5.11-3014.19 ng/mL) is delineated in this paper. A heated nebulizer in positive multiple reaction monitoring mode was employed with transitions  $m/z$  292.2 → 141.1 and 288.2 → 117.0 for terbinafine and naftifine, respectively, resulting in excellent chromatographic separation on a Hypurity Advance (50 x 4.6 mm, 5 µm) column. The developed method was successfully applied to clinical samples and for the first time demonstrated marked improved extraction efficiency and reliable long-term plasma stability results without any internal standard response variation during the entire course of study.<sup>[14]</sup>

### 2.15 BENZOIC ACID

Cho WK, Seo H et al. reported LC-MS/MS method for quantification of Benzoic acid with the title "Determination of a novel phosphodiesterase-4 inhibitor, 3-[1-(3cyclopropylmethoxy-4-difluoromethoxybenzyl)-1H-pyrazol-3-yl]-benzoic acid (PDE-423) in rat plasma using liquid chromatography-tandem mass spectrometry" is described as follows: A method for determining a novel phosphodiesterase-4 inhibitor, 3-[1-(3cyclopropylmethoxy-4-difluoromethoxybenzyl)-1H-pyrazol-3-yl]-benzoic acid (PDE-423), in rat plasma was developed and validated using liquid chromatography-tandem mass spectrometry for further pharmacokinetic study for development as a novel anti-asthmatic drug. PDE-423 in the concentration range of 0.02-10 µg/mL

was linear with a correlation coefficient of  $>0.99$ , and the mean intra- and inter-assay precisions of the assay were 7.50 and 3.86%, respectively. The validated method was used successfully for a pharmacokinetic study of PDE-423 in rats.<sup>[15]</sup>

### 2.16 CICLOPIROX

Bu W, Fan X *et al.* reported LC-MS/MS method for quantification of Ciclopirox with the title "A direct LC/MS/MS method for the determination of ciclopirox penetration across human nail plate in vitro penetration studies" is described as follows: Due to severe chelating effect caused by N-hydroxypyridone group of ciclopirox, there is no published direct HPLC or LC/MS/MS method for the determination of ciclopirox in any in vitro or in vivo matrix. Instead, the time-consuming pre-column derivatization methods have been adapted for indirect analysis of ciclopirox. After overcoming the chelating problem by using K(2)EDTA coated tubes, a direct, sensitive and high-throughput LC/MS/MS method was successfully developed and validated to determine the amount of ciclopirox that penetrated across the nail plate during in vitro nail penetration studies. The method involved adding a chemical analog, chloridazon as internal standard (IS) in K(2)EDTA coated tubes, mixing IS with ciclopirox in a 96-well plate and then proceeding to LC/MS/MS analysis. The MS/MS was selected to monitor  $m/z$  208.0- $\rightarrow$ 135.8 and 221.8- $\rightarrow$ 77.0 for ciclopirox and IS, respectively, using positive electrospray ionization. The method was validated over a concentration range of 8-256 ng/mL, yielding calibration curves with correlation coefficients greater than 0.9991 with a lower limit of quantitation (LLOQ) of 8 ng/mL. The assay precision and accuracy were evaluated using quality control (QC) samples at three concentration levels. Analyzed concentrations ranged from 101% to 113% of their respective nominal concentration levels with coefficients of variation (CV) below 10.6%. The average recovery of ciclopirox from nail matrix was 101%. The validated method was successfully used to analyze the ciclopirox formulation and in vitro nail penetration samples.<sup>[16]</sup>

### 2.17 BUTENAFINE HYDROCHLORIDE

Mitra A, Kim N *et al.* reported LC-MS/MS method for quantification of Butenafine Hydrochloride with the title "Use of an in vitro human skin permeation assay to assess bioequivalence of two topical cream formulations containing butenafine hydrochloride (1%, w/w)" is described as follows: The primary objective of this work was to investigate, using an in vitro human skin permeation study, whether changes in the excipients of butenafine hydrochloride cream would have any effect on bioperformance of the formulation. Such in vitro data would be a surrogate for any requirement of a bioequivalence (BE) study to demonstrate formulation similarity. A LC-MS/MS method for quantitation of butenafine in various matrices was developed and validated. A pilot study was performed to validate the in vitro skin permeation methodology using three cream

formulations containing butenafine hydrochloride at concentrations of 0.5, 1.0 and 1.5% (w/w). Finally, a definitive in vitro human skin permeation study was conducted, comparing the extent of butenafine hydrochloride permeation from the new formulation to that from the current formulation. The results of the study comparing the two formulation showed that there was no statistically significant difference in the extent of butenafine permeation into human skin. In conclusion, these in vitro data demonstrated that the formulation change is likely to have no significant impact on the bioperformance of 1% (w/w) butenafine hydrochloride cream.<sup>[17]</sup>

### 3. CONCLUSION

In the reported methods, Amphotericin B was analyzed after separation by solid phase extraction, total AMB (T-AMB) was determined after protein precipitation and L-AMB was determined by difference. Buparvaquone, Nystatin, and Etomidate impurity B were extracted from samples of porcine muscle, pasteurized whole milk, and eggs using 10mM ammonium formate in acetonitrile followed by liquid-liquid purification with n-hexane. In Caspofungin quantification, the DBS samples were prepared by spotting whole blood onto Whatman 903 filter paper, drying at room temperature and extracting with 50% methanol and further cleaned by protein precipitation with acetonitrile. Micafungin was analyzed in 100- $\mu$ L plasma samples over a wide range of concentrations (0.1-20  $\mu$ g/mL) by LC-MS/MS after protein precipitation. The extraction of anidulafungin from plasma by protein precipitation remained unchanged, but the changes in chromatography warranted validation of a new method, M2, 2 years after M1 was validated. In the quantification of Griseofulvin Samples were prepared using solid phase extraction and analysed without drying and reconstitution. The analytes were chromatographed on Hypersil, hypurity C18 reverse phase column under isocratic conditions using 0.05% formic acid in water:acetonitrile (30:70, v/v) as the mobile phase. In Econazole and Clotrimazole estimation, homogenized tissue samples were extracted with acetonitrile and subsequently underwent freezing-delipidation. In simultaneous quantification of Itraconazole, Hydroxy itraconazole, Keto itraconazole and N-Desalkyl itraconazole One hundred and fifty microliters of human plasma were extracted using a solid-supported liquid extraction (SLE) method and the final extracts were analyzed using reverse-phase chromatography and positive electrospray ionization mass spectrometry. In Posaconazole estimation, punched spots were then extracted by using a mixture of acetonitrile and water containing stable labeled internal standard (IS). In the estimation of Terbinafine solid-phase extraction of terbinafine achieving mean recovery of 84.3% (CV  $<$  4%) without compromising sensitivity (limit of quantitation 5.11 ng/mL) or linearity (5.11-3014.19 ng/mL) is delineated in this paper. The method was said to successfully applied to a pharmacokinetic study, to assess the bio availability and bio equivalence

studies in biological samples. Therapeutic drug monitoring of antifungal agents in biological fluids is possible with aid of the above mentioned techniques.

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