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Review Article

Separation and analysis of triazole antifungal in biological matrices by liquid chromatography: a review

Untung Gunawan[1,2], Slamet Ibrahim[3], Atthar Luqman Ivansyah[4,5], Sophi Damayanti[1,6]

1 *Department of Pharmacochemistry, School of Pharmacy, Institut Teknologi Bandung, Bandung, Indonesia*
2 *Department of Pharmacy, School of Medicine and Health Sciences, Atma Jaya Catholic University of Indonesia, Jakarta, Indonesia*
3 *Faculty of Pharmacy, Universitas Jenderal Achmad Yani, Cimahi, Indonesia*
4 *Instrumentation and Computational Physics Research Group, Department of Physics, Faculty of Mathematics and Natural Sciences, Institut Teknologi Bandung, Bandung, Indonesia*
5 *Master Program in Computational Science, Faculty of Mathematics and Natural Science, Institut Teknologi Bandung, Bandung, Indonesia*
6 *University Center of Excellence on Artificial Intelligence for Vision, Natural Language Processing & Big Data Analytics (U-CoE AI-VLB), Institut Teknologi Bandung, Bandung, Indonesia*

Corresponding author: Sophi Damayanti (sophi.damayanti@itb.ac.id)

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Abstract

Invasive fungal infections cause serious illness and death worldwide. Long-term therapeutic and preventative use of antifungal drugs in high-risk patients has caused resistance. Triazole antifungals are widely used to prevent and treat fungal infections, and therapeutic drug monitoring has been suggested to improve outcomes, reduce toxicity, and prevent drug resistance. Common methods used for monitoring triazole antifungal drugs in biological matrices such as blood, serum, and plasma include bioassay and instrumentation methods, especially liquid chromatography. Sample preparation is needed to remove interference from liquid chromatography for reliable results. This paper evaluates the use of liquid chromatography to analyze triazole antifungal agents. We provided various chromatographic techniques combined with different detector types to analyze triazole antifungal drugs in biological matrices. We also compared chromatography systems with different sample preparation methods in order to select the most suitable analytical method for bioanalysis.

Keywords

fungal infection, triazole, bioanalysis, sample preparation, liquid chromatography

Introduction

Millions of people die every year as a consequence of invasive fungal diseases. Patients with impaired immune systems frequently develop invasive fungal infections, such as those undergoing chemotherapy, organ transplantation, or other diseases that may cause an existing immune system deficiency (Pianalto and Alspaugh 2016). Aspergillosis, candidiasis, and invasive mucormycosis are all quite common; available data show yearly incidences of over 300,000, 750,000, and 10,000 cases, respectively. (Bongomin et al. 2017). Invasive fungal disease epidemiology leads to mortality rates from 30 to 95% for invasive aspergillosis and 46 to 75% for candidiasis (Brown

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et al. 2012). Fungal infections may be categorized into two types: superficial and invasive. A superficial fungal infection affects only the body's outermost layer, but an invasive fungal infection spreads throughout the body. Invasive fungal infections represent a substantial and widespread clinical concern; in high-risk groups like immunocompromised people or cancer patients undergoing chemotherapy, they have become a significant source of illness and death. *Candida* spp., *Aspergillus* spp., and *Cryptococcus* spp. are the three most common organisms that cause invasive fungal infections (Miceli et al. 2011). In clinical applications, serious fungal infections continue to be a big problem. In the past few years, notable advancements have occurred in the understanding and management of invasive fungal infections that involve aspects such as prognosis, diagnosis, and therapy. Fungal infections are becoming more common as the number of critically ill and immunocompromised people increases (Eades and Armstrong-James 2019).

Aspergillus species are potentially lethal illnesses for patients, particularly those with high-risk conditions, including stomach cancer, chronic obstructive pulmonary disease (COPD), neutropenia, hematopoietic stem cell, and organ transplantation**,** cystic fibrosis, immunodeficiency, and corticosteroid usage (Miceli et al. 2017; Fishman and Grossi 2020; Poli et al. 2020). The Infectious Diseases Society of America (IDSA) practice guidelines for the diagnosis and management of aspergillosis recommend triazole antifungals (voriconazole, itraconazole, posaconazole), amphotericin B and its liposomes, in addition to echinocandins (micafungin, caspofungin) as therapy and preventive strategy for invasive aspergillosis. It is recommended that patients receiving azole antifungal therapy undergo therapeutic drug monitoring (TDM) to prevent therapy failure caused by suboptimal doses and to reduce the resulting toxicity (Patterson et al. 2016). One of the primary causes of death among fungal illnesses is invasive candidiasis, with fifteen species causing illness in humans. Only five fungal contribute to 90% of invasive infections: *C. albicans*, *C. glabrata*, *C. krusei*, *C. tropicalis*, and *C. parapsilosis*. Because each of these pathogens has a distinct virulence, resistance, and epidemiological potential, invasive candidiasis is widely used to describe infections caused by these five pathogens. Triazole antifungals, echinocandins, flucytosine, amphotericin B, and related liposomes are recommended for invasive candidiasis treatment by IDSA: clinical practice guideline for the management of candidiasis (Pappas et al. 2015). Mucormycosis refers to an infection produced by a fungus from the order Mucorales. *Rhizopus* spp., *Mucor* spp., *Rhizomucor* spp, *Lichtheimia* spp., *Cunninghamella* spp., *Apophysomyces* spp., and *Saksenaea* spp. are frequently identified as the primary pathogens associated with mucormycosis (Roden et al. 2005; Sridhara et al. 2006; Skiada et al. 2011). Patients with mucormycosis infections require immediate treatment because the infection spreads rapidly and is dangerous to health. Delays in the identification and treatment of invasive mucormycosis are frequently correlated with increased mortality rates (Chamilos et al. 2008; Vaughan et al. 2018). As a systemic antifungal therapy for invasive mucormycosis, the European Confederation of Medical Mycology (ECMC) recommends amphotericin B and its liposomes, azole antifungals (posaconazole and isavuconazole). The recommends amphotericin B and its liposomes, and triazole antifungals (posaconazole and isavuconazole) (Cornely et al. 2019). Patients with HIV infection with a T lymphocyte count of fewer than 200 cells per L or other immunological abnormalities are susceptible to Cryptococcosis, presenting mostly as meningoencephalitis (Setianingrum et al. 2019). According to WHO guidelines, the recommended treatment approach during the induction phase involves the administration of amphotericin B and flucytosine, followed by fluconazole. The alternative treatment options encompass amphotericin B in combination with fluconazole or fluconazole combined with flucytosine. During the consolidation phase, it is suggested to provide fluconazole as the preferred treatment, similarly, in the maintenance phase or secondary prophylaxis infections. To ensure the effectiveness of treatment, reduce the risk of toxicity, and avoid the development of drug resistance, triazole antifungal drugs, commonly used to prevent and treat invasive fungal infections, must be monitored. Triazole antifungal concentration is often measured using two primary methodological methods: bioassays and instrumental approaches. Current methods for analyzing triazole antifungals, as well as the advantages and disadvantages of the method are shown in Table 1. Liquid chromatography methods have emerged as commonly used methods for quantifying antifungal compounds in biological samples. These methods utilize spectrophotometry as well as mass spectrometry as detectors. With the widespread use of methods, it is necessary to comprehend the selection of the chromatographic system to obtain an analytical method that conforms to established standards. This paper aims to review the use of liquid chromatography to analyze triazole antifungal agents using various detection methods. Various sample preparation methods were also compared to offer guidance on selecting the most suitable analytical method for the bioanalysis of triazole antifungal drugs.

Triazole antifungal drugs

Currently, four different antifungal agents are employed in managing systemic invasive fungal infections, including azoles, polyenes, echinocandins, and pyrimidine analogues (Carmona and Limper 2017). Four existing antifungal groups (Fig. 1) have different targets for fungi. The first class, known as polyene derivatives, such as amphotericin B, have the ability to interact with ergosterol, an essential part of fungal cell membranes. Amphotericin is fungicidal against *Candida* sp., *A. fumigatus*, and *A. flavus* (Meletiadis et al. 2007; Kumar et al. 2018). The second group is the first-generation and second-generation triazole antifungals, which work by interfering with the

Table 1. Current method of triazole antifungals bioanalysis.

Method	Advantages	Drawbacks
Bioassay	Easy to perform, the utilization of costly instruments is not necessary. The total antifungal activity of a drug can be determined.	Unable to quantify the individual concentrations of the drug's constituents and metabolites. Necessitates a substantial time for analysis. Poor solubility of some triazole agents in water and limited diffusion in the aqueous environment.
Spectrophotometry	Methods are simple to implement and take less time, provide greater selectivity than the bioassay technique.	It may be necessary to perform derivatization procedures prior to the detection process. Endogenous compounds may interfere with the analysis results. Not applicable for simultaneous analysis.
Gas chromatography	Short analysis time, can be used for simultaneous analysis.	The detectors are usually destructive. Need derivatization for improving the volatility. Limited choice because the methods for measuring antifungal agents have not been extensively developed.
High-performance liquid chromatography	Sensitivity is higher than in spectrophotometry. Possible to do simultaneous analysis. The detectors used are usually not destructive; therefore, the analytes may be collected for further analysis. Wide choice of detectors.	Substantial sample preparation is required, susceptibility to matrix effects. It is generally necessary to employ extended runtimes to achieve a selected analysis approach.
Ultra-high-performance liquid chromatography	Improved chromatographic efficiency compared to the HPLC method. Shorter analysis time. Less susceptible to matrix effect.	Smaller particles in the column necessitate more laborious sample processing to prevent blockage. High cost of the instrument

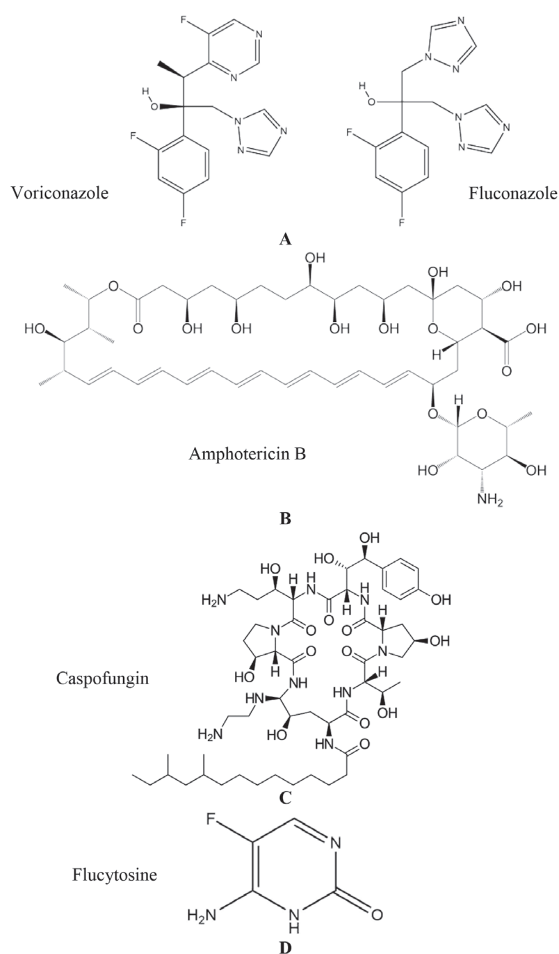

Figure 1. The chemical formula of some antifungal agents: triazoles (**A**), polyenes (**B**), echinocandins (**C**), and pyrimidine analogues (**D**).

ergosterol biosynthesis at the lanosterol demethylation stage (Geißel et al. 2018). Echinocandins, which belong to the third class of antifungal agents, work by inhibiting the production of β(1,3)-D-glucan in the cell walls of fungi. Echinocandins have fungicidal activity against Candida spp. and fungistatic activity against *Aspergillus* spp. (Patil and Majumdar 2017). Flucytosine, the fourth group interacting with the fungal cell nucleus, affects protein and DNA biosynthesis (Carmona and Limper 2017). Excessive use of antifungal compounds can increase the resistance of opportunistic pathogens (Revie et al. 2018).

The amount of nitrogen atoms in the ring is relevant for classifying azole antifungals. If the ring attaches to two nitrogen atoms, it is an imidazole group. Imidazole antifungals have no activity against Aspergillus species, so they are generally used to treat mucosal infections (Peyton et al. 2015). The triazole group has a five-ring structure that binds three nitrogen and two carbon atoms. The triazole core is prevalent in numerous compounds exhibiting pronounced antifungal, antimicrobial, and antiviral activities (Koval et al. 2022). It is currently a concern that the number of invasive fungus species resistant to antifungal agents is increasing. Antifungal triazoles with one or more 1,2,4-triazole rings have proved effective against various fungal species. Triazole antifungals work by inhibiting the CYP450 enzyme, which is involved in the production of ergosterol (Strushkevich et al. 2010). Ergosterol is required for the formation of fungal cell membranes. Treatment of fungal infections with the triazole group will result in the inhibition of ergosterol and increasing methylated sterol concentration, such as 4,14-dimethylzymosterol and 24-methylenedihydrolanosterol (Fig. 2). Accumulation of methylsterol in the membranes of fungal cells will result in cell death or growth inhibition. The triazole antifungals have different affinities for the 14-demethylase enzyme, resulting in different pharmacological effects and effects (Skiada et al. 2011; Falci and Pasqualotto 2013). Itraconazole and fluconazole are some of the first generation triazole antifungals. A decade later, the second-generation triazole antifungals, a decade later the second generation triazole antifungals including voriconazole, posaconazole, and isavuconazole were designed to overcome the shortcomings of the first-generation triazole antifungals (Peyton et al. 2015). Triazole antifungals are developing rapidly and are becoming one of the main choices in various local and systemic fungal infections due to the extensive range of activities, good pharmacokinetic parameters, and

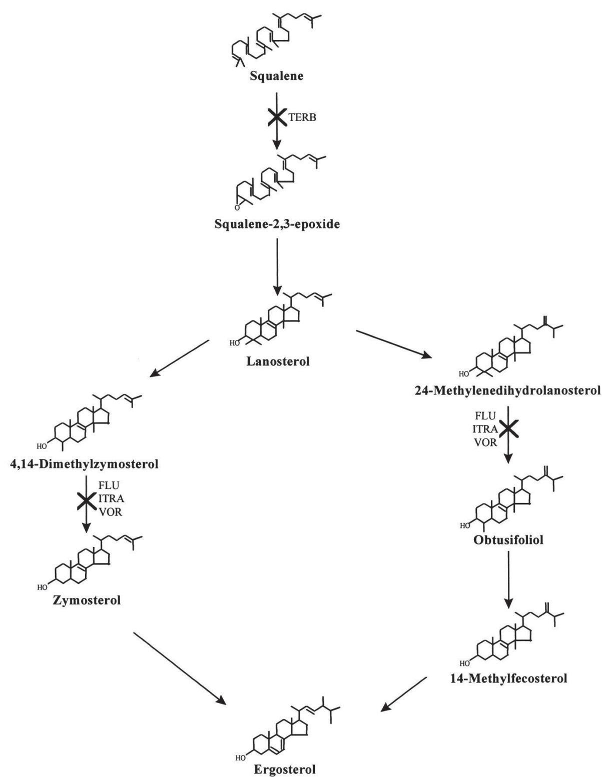

Figure 2. Antifungal agents inhibitory activities in ergosterol biosynthetic pathway (TERB: terbinafine, FLU: fluconazole, ITRA: itraconazole, VOR: voriconazole). Reuse with permission from (Ghannoum and Rice 1999).

lower side effects than amphotericin B. The azole antifungals, especially triazoles, showed good efficacy in various fungal species with a better safety profile than amphotericin B and imidazole groups. Various synthetic routes for azole antifungals also provide an economic impact for the treatment of fungi with this class, and the discovery of novel fungus species has prompted the growth of the antifungal drug market in recent years (Wang et al. 2009; Montagna et al. 2013).

Therapeutic drug monitoring of triazole antifungal

Therapeutic drug monitoring (TDM) is a multidisciplinary approach predominantly used to prevent or reduce adverse drug effects in patients. TDM has been used widely for narrow therapeutic index drugs (Caro et al. 2020). TDM leads to enhanced patient treatment efficacy and safety. A relationship between drug levels (pharmacokinetics) and pharmacological activity (pharmacodynamics) must be implemented for TDM to be effective, and the efficacy or toxicity must be well-defined (Kuhlin et al. 2019). The following are the primary indications for using TDM: abnormal response to therapy, unusual toxicity, abnormalities in hepatic or renal function and impaired metabolism (such as pregnant women, kids, the elderly, and the obese), narrow therapeutic range drug for treatment, assessment of an insufficient relationship between dose and clinical response; inability to monitor the patient's progress by physical examination or standard laboratory analysis, inter-and intra-individual variability of metabolization (Esteve-Romero et al. 2016; Tuzimski and Petruczynik 2020). Antifungal drugs need to meet several criteria in TDM for the results to be clinically useful. First, there must be sensitive analytical methods in the laboratory that can report the results in a short time so that it helps clinical decision-making. Second, To maximize the effectiveness of its treatment and minimize the risk of toxicity, the antifungal drug must have a therapeutic range. Finally, the compound must not have significant inter-patient variability to not cause harm. Triazole antifungal agents have been employed in TDM for the treatment of invasive fungal infections and fulfill established criteria (David et al. 2009; Ashbee et al. 2014). TDM approaches tend to depend significantly on accurate and objective analytical techniques, especially for low sample concentrations. In addition, the selection of an effective analytical method requires automation, high-throughput instrumentation, robustness, and affordable costs. For many years, immunoassay was implemented for TDM; however, immunoassay lacks specificity in analyte recognition due to interference from related substances, metabolites, and matrices. These days, the majority of laboratories employ liquid chromatography in conjunction with ultraviolet (UV), fluorescence (FLD), and mass (MS) detectors, which are highly accurate, sensitive, and precise analytical methods for TDM. When combined with selective detection techniques, liquid chromatography has excellent analytical performance for separating analytes from other compounds and metabolites present in biological matrices. Selecting the proper sample preparation method, column type, internal standard, and detection conditions is essential for achieving accurate drug measurements and minimizing matrices or metabolite interference (Tuzimski and Petruczynik 2020).

Analytical method

The analytical method consists of three main steps, including sampling, sample preparation, and sample measurement. Sample preparation aims to reduce interference from the matrices that can interfere with the analysis process, enrich the sample, transform the analyte into an appropriate form, and improve measurement reproducibility (Hu et al. 2013). Triazole antifungals can be analyzed by various methods, such as titrimetric methods and instrument methods, such as spectrophotometry, electrochemistry, and chromatography. Titrimetric and spectrophotometric techniques cannot be used to analyze triazole antifungals in complex matrices, such as biological matrices, due to selectivity limitations (Ekiert et al. 2010). Nowadays, High-Performance Liquid Chromatography (HPLC) and Ultra Performance Liquid Chromatography (UPLC) are the most commonly used and developed

analytical techniques for determining triazole antifungal concentration in biological matrices, where plasma and serum samples were used in most documented protocols.

HPLC-Ultraviolet detector

Despite the fact that liquid chromatography with a mass detector (LC-MS) is the preferred method for quantifying triazole antifungals in biological matrices, its limitations, such as susceptibility to matrix effects, the need for trained operators, and high operating costs, imply that not all laboratories working on drug analysis have LC-MS technology. In contrast to LC-MS, the widespread use of HPLC with a UV detector in clinical labs does not require the employment of expensive equipment or highly educated employees. HPLC-UV and photodiode array (PDA) are still essential as a simple, affordable, and conveniently accessible analytical method for measuring triazole antifungal in biological matrices (Zarad et al. 2021). Due to the capability of the PDA detector to collect the whole spectrum at each time point, the chromatogram is more informative and selective than those with only one wavelength. UV spectra are commonly utilized in combination with retention data to correctly identify compounds in biological samples where their identities are unknown or suspected. HPLC-UV and PDA have several benefits, including high specificity, sensitivity, speed, and durability. The obtained information, which contains the retention time and absorption spectra of the analyte substances provides excellent identification power that is inexpensive and widely available to analytical laboratories (Ashbee et al. 2014). To enhance the sensitivity and selectivity of the HPLC-UV method, sample preparation was routinely optimized to achieve excellent analyte recoveries and minimize matrices interferences (Nannetti et al. 2018). Choudary utilized HPLC-UV to simultaneously determine triazole antifungals (voriconazole, itraconazole, and posaconazole) in human serum (Choudhary et al. 2021). The method required uncomplicated sample preparation by protein precipitation with acetonitrile, followed by centrifugation, and then the sample was analyzed in the HPLC system. The simultaneous analysis was conducted using the C_{18} column, elution using isocratic mode, a mobile phase composed of acetonitrile and water, and detection was achieved at 266 nm for posaconazole, itraconazole, and it's metabolite, 255 nm for voriconazole. The linearity result showed that the correlation coefficient of itraconazole, voriconazole, and posaconazole >0.999, limit of detection (LoD) of itraconazole and voriconazole were 0.25 mg/L, posaconazole was 0.125 mg/L. Limit of quantification (LoQ) was 0.5 mg/L for itraconazole and voriconazole, 0.25 mg/mL for posaconazole. Precision and accuracy were within acceptable limits, with a 100 percent average percentage recovery, and there were no interferences from the endogenous matrices or other antimicrobial agents observed in the chromatogram. Simultaneous analysis of voriconazole, itraconazole, posaconazole, and its metabolite had been developed by Lopez using HPLC with a PDA detector (Gomez-Lopez et al. 2018). Identification of the sample was performed at three distinct wavelengths (255, 266, and 311 nm). Simple extraction by protein precipitation using acetonitrile, followed by centrifugation, was carried out to eliminate matrices interferences. For all substances tested, the assay demonstrated a linearity range from 0.25 to 16 mg/L. The method was accurate and precise, both intra and inter-day precision. Further applications of HPLC with UV and DAD detectors for the analysis of triazole antifungal in biological matrices are shown in Table 2.

HPLC-Fluorescence detector

HPLC with a fluorescence detector (FLD) provided some advantages compared to UV detectors. It's more selective and sensitive than UV detectors, so HPLC-FLD is applicable for analyzing targeted analytes in biological matrices. When analyzing biological samples with low analyte concentrations, the sensitivity of HPLC-FLD is crucial since it is around 30 times greater than UV detectors (Tuzimski and Petruczynik 2020). Triazole antifungal drugs were infrequently studied using HPLC with fluorescence detection as opposed to UV or MS detectors. An HPLC-FLD method for the determination of posaconazole concentrations in human plasma and serum was developed and validated by Tang (2017). The samples were prepared by protein precipitation in methanol, and the analysis was performed on a C18 column using a mobile phase of ammonium acetate, water, and acetonitrile for 8 minutes run time. The excitation wavelength for detection was 245 nm, while the emission wavelength was 380 nm. The technique has a linear response from 0.1 to 10 g/mL, with LOD and LOQ 0.04 and 0.01 μg/mL. Pharmacokinetic and TDM study of free and total voriconazole using HPLC-FLD was accomplished using isocratic system elution HPLC with acetonitrile potassium dihydrogen phosphate buffer was used for sample separation (Resztak et al. 2020). One-step sample preparation by precipitating proteins was performed for the extraction of voriconazole from the plasma. A linear standard calibration curve was obtained for free VCZ across the concentration range of 0.05–10.0 g/mL (Fig. 2), while a linear calibration curve was obtained for total VCZ over the concentration range of 0.1–10.0 g/mL. Table 3 displays various uses of HPLC-FLD in the analysis of triazole antifungals in biological matrices.

HPLC-mass detector

Triazole antifungal drug detection in biological matrices is most commonly performed using liquid chromatography with a mass detector (MS). Compared to tandem mass spectrometry, a single MS lacks sensitivity and selectivity. To optimize the therapeutic effectiveness of TDM, accurate, precise, and rapid quantitative methods are necessary. The approach of LC combined with tandem MS enables the

Table 2. Analysis of triazole antifungal drugs using HPLC-UV/PDA. NA = not available.

No	Analyte	Matrices	Sample Preparation	HPLC system	Stationary Phase	Mobile Phase	LOD/LLOD	LOQ/LLOQ	References
1	Voriconazole, posaconazole, itraconazole	Human serum	Protein precipitation using acetonitrile, and centrifugation	HPLC-PDA, detection at 255&262 nm	C18 (250 × 4.6 mm, 5 µm)	Isocratic mode, acetonitrile:water (70:30), flow rate 1.0 mL/min	0.25 ug/mL for voriconazole and Itraconazole, 0.125 µg/mL for posaconazole	0.5 ug/mL for voriconazole and Itraconazole, 0.25 µg/L for posaconazole	(Choudhary et al. 2021)
2	Voriconazole	Human serum	Protein precipitation using acetonitrile, vortex, and centrifugation	HPLC-UV, detection at 255 nm	C18 (250 × 4 mm, 5 µm)	Isocratic mode, acetonitrile:water (60:40), flow rate 0.8 mL/min	0.125 µg/mL	0.25 µg/mL	(Blanco-Dorado et al. 2021)
3	Voriconazole	Human plasma	Protein precipitation using methanol, vortex, and centrifugation.	HPLC-UV, detection at 256 nm	C18 (250 × 4.6 mm, 3.5 µm)	Gradient mode, 0.05 M ammonium acetate, acetonitrile, and methanol, flow rate 1 mL/min	0.042 µg/mL	0.125 µg/mL	(Yousefian et al. 2021)
4	Fluconazole	Human serum	SPE protein-coated (PC) µBondapak CN silica column (PC-µB-CN-column)	HPLC-UV, detection at 260 nm	C18 (150 × 4 mm, 5 µm)	Isocratic mode, acetonitrile:water (20:80), flow rate 1 mL/min	0.05 µg/mL	0.18 µg/mL	(Zarad et al. 2021)
5	Fluconazole	Cerebrospinal fluid	Dispersive liquid-liquid microextraction using chloroform, isopropyl alcohol, and phosphate buffer pH 7.3	HPLC-PDA, detection at 210 nm	C18 (100 × 4.6 mm, 2.7 µm)	Isocratic mode, ethanol:water (15:85), flow rate 0.8 mL/min	NA	0.25 µg/mL	(Moreira et al. 2020)
6	Voriconazole, fluconazole	Rat plasma	SPE using metal organic framework	HPLC-UV, detection at 210 nm	C18	Isocratic mode, methanol: water (60:40), flow rate 1.0 mL/min	0,03 µg/mL for voriconazole and 0.02 µg/mL for fluconazole	0,05 µg/mL for voriconazole and 0.04 µg/mL for fluconazole	(Bashir et al. 2020)
7	Isavuconazole	Human plasma	Protein precipitation using methanol, vortex, and centrifugation.	HPLC-PDA, detection at 259 nm	C18 (150 × 4.6 mm, 3.5 µm)	Gradient mode, acetonitrile, phosphate buffer pH 4.5, flow rate 1 mL/min	NA	0,4 µg/mL	(Cozzi et al. 2018)
8	Voriconazole, itraconazole, and posaconazole	Human serum	Protein precipitation using acetonitrile	HPLC-PDA, detection at 255, 266, and 311 nm)	C18 (150 × 4.6 mm, 5 µm)	Gradient mode, acetonitrile and water, flow rate 1 mL/min	0.125 µg/L for voriconazole, itraconazole, and posaconazole	0.25 µg/L for voriconazole, itraconazole, and posaconazole	(Gomez-Lopez et al. 2018)
9	Isavuconazole	Human plasma	Protein precipitation using acetonitrile, followed by SPE	HPLC-PDA, detection at 285 nm	C18 (150 × 4.6 mm, 3.5 µm)	Isocratic mode, ammonium acetate buffer pH 8.0 and acetonitrile (45:55), flow rate was 1.0 mL/min	0.012 µg/mL	0.025 µg/mL	(Nannetti et al. 2018)
10	Voriconazole	Human Serum	Protein precipitation using acetonitrile, vortex, and centrifugation	HPLC-PDA, detection at 262 nm	C18 (125 × 4.6 mm, 5 µm)	Isocratic mode, acetonitrile:water (40:60), flow rate 0.4 mL/min	0.125 µg/mL	0.25 µg/mL	(Badiee et al. 2017)
11	Terconazole, voriconazole, posaconazole, ravucunazole, itraconazole	Human plasma and urine	Protein precipitation using trichloroacetic acid, followed by microextraction-packed sorbent	HPLC-PDA, detection at 210 nm	C18 (250 × 4.6 mm, 5 µm)	Gradient mode, phosphate buffer pH 2.5 and acetonitrile, flow rate at 1.0 mL/min	0.007 µg/L for ravuconazole, 0.07 µg/L for terconazole, and 0.017 µg/L for voriconazole, posaconazole, and itraconazole	0.02 µg/L for ravuconazole, 0.2 µg/L for terconazole, and 0.05 µg/L for voriconazole, posaconazole, and itraconazole	(Campestre et al. 2017)
12	Voriconazole	Human plasma	Protein precipitation using perchloric acid, vortex, and centrifugation.	HPLC-PDA, detection at 254 nm	C18 (100 × 2.0 mm, 2.2 µm)	Isocratic mode, propylene carbonate: (70% NaH_2PO_4 pH = 3.0) + 30% EtOH) (10:90), flow rate of 0.3 mL/min	0.05 µg/mL	0.5 µg/mL	(Dogan and Basci 2017)
13	Terconazole, voriconazole, posaconazole, ravucunazole, itraconazole	Human plasma	Fabric Phase Sorptive Extraction	HPLC-PDA, detection at 210 nm	C18 (250 × 4.6 mm, 5 µm)	Gradient mode, phosphate buffer pH 2.5 and acetonitrile, flow rate at 1.0 mL/min	0.03 µg/mL	0.1 µg/mL	(Locatelli et al. 2017)
14	Voriconazole	Human plasma	Protein precipitation using perchloric acid, vortex, and centrifugation.	HPLC-UV, detection at 255 nm	C18 (250 × 4.6 mm, 5 µm)	Isocratic mode, acetonitrile:water (7:3), flow rate 1 mL/min	NA	0,2 µg/mL	(Chawla et al. 2016)
15	Voriconazole, posaconazole	Human plasma	Protein precipitation using acetonitrile, vortex, and centrifugation.	HPLC-UV, detection at 250 nm	C18 (250 × 4.6 mm, 5 µm)	Isocratic mode, water:methanol:acetonitrile (35:15:50), flow rate 1.0 mL/min	0.05 µg/mL for voriconazole, 0.02 µg/mLfor posaconazole	0.1 µg/mL for voriconazole, 0.03 µg/mLfor posaconazole	(Francia 2015)
16	Posaconazole	Rat plasma	LLE using diethyl ether in sodium hydroxide	HPLC PDA, detection at 220 nm	C18 (250 × 4.6 mm, 5 µm)	Gradien mode, acetonitrile and potassium dihydrogen orthophosphate, flow rate 1.5 mL/min	NA	0.05 µg/mL	(Khalil et al. 2015)

No	Analyte	Matrices	Sample Preparation	HPLC system	Stationary Phase	Mobile Phase	LOD/LLOD	LOQ/LLOQ	References
17	Fluconazole	Human plasma	Protein precipitation using acetonitrile and NaCl, centrifugation	HPLC-UV, detection at 261 nm	C8 (125 × 4.0 mm, 5 μm)	Isocratic mode, acetonitrile:potassium dihydrogen phosphate buffer (15:85), pH 3.0, flow rate of 1.5 mL/min	0.02 μg/mL	0.061 μg/mL	(Safaei et al. 2015)
18	Itraconazole	Human plasma	Protein precipitation using acetonitrile, vortex, and centrifugation	HPLC-UV, detection at 258 nm	CN (150 × 3.9 mm, 5 μm)	Isocratic mode, sodium dodecyl sulfate, 1-propanol, triethylamine in o-phosphoric acid, flow rate 2.0 mL/min	5.4 μg/mL	16.4 μg/mL	(Rizk et al. 2014)
19	Voriconazole	Human serum and plasma	Protein precipitation using methanol, vortex, and centrifugation	HPLC-UV, detection at 256 nm	C18 (250 × 4.6 mm, 5 μm)	Isocratic mode, ammonium acetate:acetonitrile:methanol (40:20:40), flow rate 1.0 mL/min	0.06 μg/mL	0.1 μg/mL	(Tang 2013)
20	Voriconazole, posaconazole, and itraconazole	Human plasma	Protein precipitation using perchloric acid and methanol, centrifugation.	HPLC-UV, detection at 262 nm	C6 (150 × 4.6 mm, 5 μm)	Gradient mode, phosphate buffer pH 3.5, acetonitrile, and water, flow rate 1.0 mL/min	NA	0.05 mg/L for voriconazole, posaconazole, and itraconazole	(Zhang et al. 2013)
21	Fluconazole	Human plasma	Protein precipitation using sodium hydroxide and dichloromethane, vortex, and centrifugation.	HPLC-UV, detection at 260 nm	C18 (250 × 4.6 mm, 5 μm)	Isocratic mode, sodium acetate buffer:acetonitrile (80:20), flow-rate 1.2 mL/min	NA	0.125 μg/mL	(Liew et al. 2012)
22	Voriconazole	Human plasma	Protein precipitation using acetonitrile, vortex, and centrifugation.	HPLC-UV, detection at 255 nm	C18 (100 × 4.6 mm, 2.3 μm)	Isocratic mode, acetonirile:methanol:phosphate buffer (25:10:65), flow rate 1.5 mL/min	NA	0.1 μg/mL	(Yamada et al. 2012)
23	Voriconazole	Human serum	SPE with acetonitrile/ methanol (90:10)	HPLC-PDA, detection at 254 nm	C18 (75 × 4.6 mm, 3 μm)	Gradient mode, potassium dihydrogen phosphate, TEA, and an acetonitrile, flow rate 1.2 mL/min	0.078 μg/L	0.25 μg/L	(Zufía et al. 2010)
24	Fluconazole	Human plasma	Protein precipitation using sodium hydroxide and dichloromethane, vortex, and centrifugation.	HPLC-UV, detection at 210 nm	CN (150 × 6.0 mm, 5 μm)	Isocratic mode, water:acetonitrile (60:40), flow rate 0.5 mL/ min	0.2 μg/mL	0.4 μg/mL	(Santos et al. 2010)
25	Voriconazole	Human serum	Protein precipitation using hexane and dichloromethane, centrifugation.	HPLC UV, detection at 250 nm	C8 (250 × 4.6 mm, 5 μm)	Isocratic mode, sodium potassium phosphate buffer pH 6.0, acetonitrile, and water (45:52.5:2.5), flow rate 0.8 mL/min	0.1 mg/L	0.2 mg/L	(Steinmann et al. 2011)
26	Itraconazole	Human plasma	Protein precipitation using zinc sulfate and acetonitrile, centrifugation.	HPLC UV, detection at 263 nm	C18 (150 × 4.6 mm, 5 μm)	Isocratic mode, methanol:water (75:25), flow rate 1.0 mL/min	NA	2 μg/mL	(Shimoeda et al. 2010)

Table 3. Analysis of triazole antifungal drugs using HPLC-FLD.

No	Analyte	Matrices	Sample Preparation	HPLC system	Stationary Phase	Mobile Phase	LOD/ LLOD	LOQ/ LLOQ	References
1	Voriconazole	Human plasma	Protein precipitation using acetonitrile, centrifugation	HPLC-FLD, excitation at 254 nm, emission at 385 nm and 450 nm	C18 (125 × 4 mm, 5 μm)	Isocratic mode, acetonitrile and 10 mM potassium dihydrogen phosphate buffer (35:65), flow rate 1.2 mL/min	NA	0.1 μg/mL	(Resztak et al. 2020)
2	Isavuconazole	Human plasma	Protein precipitation using chromsystems reagent	HPLC-FLD, excitation at 261 nm, emission at 366 nm	C18	Isocratic mode, ChromSystem mobile phase, flow rate 1.0 mL/min	NA	0.15 mg/L	(Mueller et al. 2018)
3	Posaconazole	Human plasma and serum	Protein precipitation using methanol, centrifugation	HPLC-FLD, excitation at 245 nm, emission at 380 nm	C18 (250 × 4 mm, 5 μm)	Isocratic mode, ammonium acetate: water:acetonitrile:TFA (409:590:1, flow rate 1.1 mL/min	0.04 μg/mL	0.1 μg/mL	(Tang 2017)
4	Itraconazole	Human plasma	Protein precipitation using methanol, centrifugation	HPLC-FLD, excitation at 262 nm, emission at 365 nm	C18 (150 × 4 mm, 5 μm)	Isocratic mode, phosphate buffer pH 6.1: acetonitrile (35:65), flow rate of 1 mL/min	NA	NA	(Kumar et al. 2015)
5	Posaconazole, itraconazole	Human plasma and serum	protein precipitation using Tris and MTBE, centrifugation	HPLC-FLD, excitation at 260 nm, emission at 350 nm	C6 (100 × 3.0 mm, 3 μm)	Gradient mode, formic acid, methanol, flow rate 0.7 mL/min	0.05 mg/L	0.3 μg mL	(Buckner et al. 2011)

rapid and selective measurement of simultaneous triazole antifungal agents. The majority of the proposed method for chromatographic analysis of triazole antifungal medicines are used by MS detectors (Zheng and Wang 2019, Tuzimski and Petruczynik 2020). LC-MS/MS method for therapeutic drug monitoring of voriconazole, itraconazole, and posaconazole was performed (Yoon et al. 2019). Validation of the method was carried out on the linearity, accuracy, precision, carryover, and matrices effects. It took 3.8 minutes to analyze each sample (Fig. 3). Some methods of LC-MS that have been used in the determination of triazole antifungal in biological matrices are shown in Table 4.

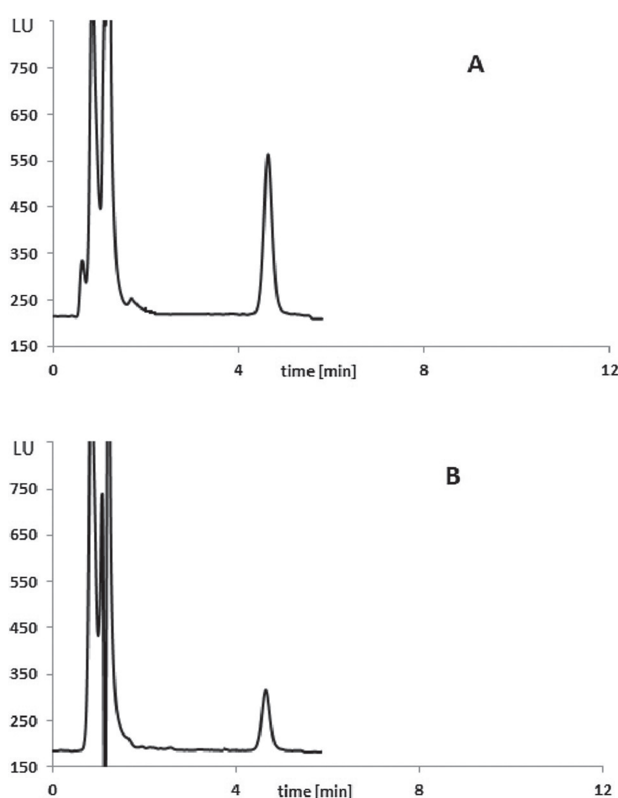

Figure 3. Chromatograms of voriconazole standard (5.0 µg/mL) in phosphate-buffered saline (**A**) and plasma of patients (**B**). Reuse with permission from (Resztak et al. 2020).

Ultra-high-performance liquid chromatography

Recently, ultra-high-pressure liquid chromatography (UPLC) has become the preferred HPLC platform. In terms of analytical time, UPLC is excellent for rapid method development due to its shorter analysis times (Fig. 4) and fast column equilibration (Dong and Zhang 2014). UPLC outperforms traditional HPLC in terms of performance (reduced system dispersion and dwell volumes) and is especially appealing for method development settings that require fast run time and rapid responsiveness to changes in column/mobile-phase conditions. A 3-to-10-fold time saving is commonly obtained using UPLC while keeping a high level of performance in resolution, sensitivity, and accuracy. Improvements in HPLC performance have prompted both revolutionary and evolutionary changes in UPLC technology (Dong and Guillarme 2013). UPLC with various detectors has been developed to simultaneously analyze triazole antifungals. The triazole antifungals: fluconazole, itraconazole, voriconazole, posaconazole, and isavuconazole were all simultaneously determined in human plasma (Tanaka et al. 2022) using UPLC with MS/MS detector, SPE was used for sample preparation, C18 column was used for separation using gradient elution consist of formic acid, acetonitrile, ammonium formate. The separation required six minute run time and provided excellent results with all validation criteria, which fulfilled US-FDA bioanalytical method validation guidance. Because of its sensitivity and specificity, as well as its ease of sample preparation and fast analysis time, the UPLC-MS technique has proven to be superior to other analytical methods. Another analysis of triazole antifungal in biological matrices using UPLC is shown in Table 5.

Sample preparation method

Blood, plasma, and serum are the most often used matrices for TDM. Recently, dried blood spots (DBS) and saliva have also been developed for TDM. Matrices such as cerebrospinal fluid (CSF), inflammatory fluids, and particular cells and tissues are not typically employed for TDM but may be useful in some conditions. Each of the biological matrices has benefits and limitations in TDM, and the clinical interpretation of the data is highly dependent on the matrices (Wong et al. 2014). Complex matrices, such as biological fluids, such as serum and plasma, and tissues containing proteins, lipids, salts, and metabolites with equivalent properties to the target analyte. Due to the rapid degradation of column frits and stationary phases, which cannot be avoided, direct injection into a chromatographic system is inadvisable. Various pre-treatment and extraction techniques may be implemented to overcome this problem based on the complexity of the matrices (Ramos 2012). The sample preparation process will be determined by the type of sample matrices. Prior to analysis, at least protein and other interfering components should be separated from the sample during sample preparation so that they do not impact the result of sample analysis. The physicochemical characteristics of the studied analyte and its metabolites will determine sample preparation techniques (Acquavia et al. 2021). Sample preparation is the method of separating target analyte from sample matrices with suitable form by physical, chemical, or biological properties before qualitative and or quantitative analysis. Before analysis, extraction and separation of target analytes, as well as the concentration of trace level chemicals, must be completed to remove matrices influence. The optimal sample preparation procedure for detecting an analyte in biological matrices should achieve optimal recoveries., eliminate potentially interfering endogenous molecules, and be rapid, simple, and inexpensive (Xia et al. 2020). Protein precipitation, liquid–liquid extraction, solid-phase extraction, or a combination of two or more of these techniques that involve analytes extraction, clean-up, and concentration prior to chromatographic separation is commonly used to prepare biological samples (Zheng and Wang 2019).

Protein precipitation

Protein precipitation (PP) is the most basic and widely used procedure for preparing samples for biological matrices. PP is most often induced by the addition of organic solvents to blood, plasma, or serum, which modifies their solvation in water. Using centrifugation, protein precipi-

Table 4. Analysis of triazole antifungal drugs using LC-MS.

No	Analyte	Matrices	Sample Preparation	HPLC system	Stationary Phase	Mobile Phase	LOD/ LLOD	LOQ/LLOQ	References
1	Voriconazole	Human serum	Protein precipitation using methanol and acetonitrile, centrifugation	LC-MS/MS, triple quadrupole with +ESI	C18 (50 × 2.1 mm, 1.6 μm)	Gradient mode, ammonium acetate in formic acid solution, acetonitrile, flow rate 0.6 mL/ min	NA	0.5 μg/mL	(Lu et al. 2022)
2	Itraconazole	Human plasma	Protein precipitation using acetonitrile vortex centrifugation	LC-MS/MS, triple quadrupole with +ESI	C18 (75 × 2.0 mm, 3 μm)	Isocratic mode, acetonitrile: ammonium acetate pH 6.0 (57:43), flow rate 0.2 mL/min	NA	0.015 μg/mL	(Imoto et al. 2020)
3	Voriconazole, itraconazole, and posaconazole	Human serum	Protein precipitation using methanol and acetonitrile, vortex, and centrifugation	LC-MS/MS, triple quadrupole with +ESI	C18 (50 × 2.1 mm, 3 μm)	Gradient mode, ammonium acetate in formic acid, acetonitrile containing formic acid, flow rate 0.5 mL/min	NA	0.1 μg/mL for voriconazole, 0.05 μg/ mL for itraconazole and posaconazole	(Yoon et al. 2019)
4	Voriconazole	Human plasma	Protein precipitation using methanol, vortex, and centrifugation	LC-MS/MS, triple quadrupole with +ESI	C18 (100 × 2.1 mm, 3.5 μm)	Gradient mode, formic acid in water and methanol, flow rate 0.4 mL/min	NA	0.1 μg/mL	(Mei et al. 2019)
5	Voriconazole	Human plasma	Protein precipitation, centrifugation	LC-MS, quadrupole with +ESI	C18 (150 × 4.6 mm, 5 μm)	Gradient mode, fomic acid in water and acetonitrile with formic acid, flow rate 1 mL/min	0.019 μg/mL	0.039 μg/mL	(Allegra et al. 2018)
6	Voriconazole	Human serum	Protein precipitation using methanol and acetonitrile, centrifugation	LC-MS/MS, triple quadrupole with +ESI	C18 (50 × 2.1 mm, 5 μm)	Gradient mode, acetic acid, ammonium acetate, trifluoroacetic acid, acetonitrile, flow rate 0.5 mL/min	NA	0.1 μg/mL	(Avest et al. 2018)
7	Voriconazole	Human plasma	SPE	LC-MS/MS, triple quadrupole with +ESI	C18 (50 × 2.1 mm, 3.5 μm)	Gradient mode, water containing formic acid:acetonitrile containign formic acid, flow rate 0.25 mL/ min	NA	0.05 μg/mL	(Martial et al. 2018)
8	Fluconazole, itraconazole, isavuconazole, posaconazole and voriconazole	Human plasma	Protein precipitation using acetonitrile, centrifugation	LC-MS, quadrupole with +ESI	C18 (150 × 4.6 mm, 5 μm)	Gradient mode, water containing formic acid:acetonitrile containign formic acid, flow rate 0.25 mL/ min	NA	58.59 ng/mL for fluconazole, 31.25ng/mL for itraconazole, 31.25 ng/mL for isavuconazole, 31.25 ng/mL for posaconazole and 58.59 ng/mL for voriconazole	(Fatiguso et al. 2017)
9	Voriconazole	Human Serum	Protein precipitation using methanol and acetonitrile, centrifugation	LC-MS/MS, triple quadrupole with +ESI	C18 (50 × 3 mm, 2.7 μm)	Isocratic mode, water:acetonitrile containing formic acid (30:70), flow rate 0.3 mL/min	NA	0.7 μg/mL	(Jeon et al. 2017)
10	Voriconazole	Human plasma	Protein precipitation using acetonitrile, centrifugation	LC-MS/MS, triple quadrupole with +ESI	C18 (50 × 4.6 mm, 2.7 μm)	Gradient mode, formic acid:acetonitrile, flow rate 0.9 mL/min	NA	0.3 μg/mL	(Li et al. 2017)

tates are then separated from the analyte target. Because of its inexpensive cost and limited method requirements, this technique is one of the most commonly used for biological matrices (Ma et al. 2008). Lipids, phospholipids, fatty acids, and another endogenous components in biological matrices usually not adequately separated from the sample because of the limitation of sample preparation using protein precipitation. This endogenous component can interfere with analysis, especially with LC-MS detection (Koster et al. 2013). PP with acetonitrile and methanol is the most commonly used preparation method in the bioanalysis of triazole antifungal. When combined with vortex and centrifugation at high speed, it can be used to separate the protein in the biological matrices (Tang 2017; Mueller et al. 2018; Blanco-Dorado et al. 2021; Yousefian et al. 2021).

Liquid-liquid extraction

One of the earliest sample preparation procedures utilized for biological sample analysis was liquid-liquid extraction (LLE). The octanol-water partition coefficient method is used in LLE to migrate analytes from an aqueous sample to a solvent that is immiscible with water. Emulsion formation, the need for extensive sample amounts, and the potential danger of organic solvents are only some of the problems with conventional LLE. Furthermore, this process sometimes requires several difficult-to-automate procedures. The LLE approach was integrated with other methods to overcome these limitations, including liquid phase microextraction (LPME) (D'Ovidio et al. 2022). LPME is the method of preparation sample, whereas the analyte target, which is usually in an aqueous solvent, sample separated with a low-volume extraction solvent, typically organic solvent. Based on the extraction solvent's interaction with the analyte, there are three forms of LPME: single drop microextraction (SDME), hollow fiber liquid-phase microextraction (HF-LPME), and dispersive liquid-liquid microextraction (DLLME) (Manousi and Samanidou 2021). LLE with some organic solvents, such as diethyl ether, methyl tert-butyl ether (MTBE), n-hexane, and dichloromethane, were used for the separation of triazole antifungal in biological matrices (Verweij-van Wissen et al. 2012; Khalil et al. 2015; Al-Ghobashy et al. 2018).

Table 5. Analysis of triazole antifungal drugs using UPLC.

No	Analyte	Matrices	Sample Preparation	HPLC system	Stationary Phase	Mobile Phase	LOD/LLOD	LOQ/LLOQ	References
1	Fluconazole, itraconazole, voriconazole, posaconazole, Isavuconazole	Human plasma	SPE using methanol, water, formic acid, ammonium hydroxide	UPLC-MS/MS, triple quadrupole with +ESI	C18 (50 × 2.1 mm, 1.7 μm)	Gradient mode, formic acid, acetonitrile, ammonium formate, flow rate 0.6 mL/min	NA	0.1 μg/mL for fluconazole, 20 ng/mL for itraconazole, 20 ng/mL for voriconazole, 5 ng/mL for posaconazole, and 50 ng/mL for isavuconazole	(Tanaka et al. 2022)
2	Posaconazole	Rat plasma	Protein precipitation using acetonitrile, vortex, and centrifugation.	UPLC-MS/MS, triple quadrupole with +ESI	C18 (100 × 2.1 mm, 1.7 μm)	Gradient mode, formic acid and acetonitrile, flow rate 0.3 mL/min	NA	5 ng/mL	(Yang et al. 2021)
3	Fluconazole, voriconazole, posaconazole	Human plasma	Protein precipitation using acetonitrile and methanol, vortex, and centrifugation.	UPLC-MS/MS, triple quadrupole with +ESI	C18 (50 × 2.1 mm, 1.7 μm)	Gradient mode, formic acid, ammonium formate, water, acetonitrile, flow rate 0.4 mL/min	NA	0.2 μg/mL for fluconazole, 0.02 μg/mL for voriconazole, 0.005 μg/mL for posaconazole	(Kai et al. 2021)
4	Voriconazole, itraconazole and fluconazole	Rat plasma	Protein precipitation using acetonitrile, vortex, and centrifugation.	UPLC-MS/MS, triple quadrupole with +ESI	C18 (50 × 2.1 mm, 1.7 μm)	Gradient mode, acetonitrile and formic acid, flow rate 0.4 mL/min	NA	0.5 ng/mL	(Xie et al. 2020)
5	Voriconazole	Human whole blood	Volumetric Absorptive Microsampling using acetonitrile and methanol	UPLC-MS/MS, triple quadrupole with +ESI	Pentafluorophenyl (50 × 4.6 mm, 2.6 μm)	Gradient mode, ammonium acetate, formic acid, acetonitrile, flow rate 0.7 mL/min	1.25 ng/mL	10 ng/mL	(Moorthy et al. 2019)
6	Voriconazole	Rat plasma	Protein precipitation using acetonitrile, vortex, and centrifugation.	UPLC-MS/MS, triple quadrupole with +ESI	C18 (50 × 2.1 mm, 1.7 μm)	Gradient mode, acetonitrile and formic acid, flow rate 0.4 mL/min	NA	5 ng/mL	(Xu et al. 2019)
7	Voriconazole	Human plasma	LLE using MTBE, centrifugation	UPLC-MS/MS, triple quadrupole with +ESI	C18 (50 × 4.6 mm, 1.7 μm)	Isocratic mode, acetonitrile:water: methanol (70:25:5), flow rate 0.3 mL/min	NA	1 ng/mL	(Al-Ghobashy et al. 2018)
8	Voriconazole	Human serum	Protein precipitation using acetonitrile, vortex, and centrifugation.	UPLC-PDA, detection at 256 nm	C18 (100 × 2.1 mm, 1.8 μm)	Gradient mode, water and acetonitrile, flow rate 0.4 mL/min	NA	0.5 μg/mL	(Bressán et al. 2018)
9	Isavuconazole	Human plasma	Protein precipitation using methanol, vortex, and centrifugation.	UPLC-MS/MS, triple quadrupole with +ESI	Pentafluorophenyl (50 × 2.1 mm, 2.6 μm)	Gradient mode, isopropanol, formic acid, ammonium acetate, flow rate 0.6 mL/min	NA	0.53125 μg/mL	(Hösl et al. 2018)
10	Isavuconazole	Human plasma	Protein precipitation using ChromSystems reagent, vortex, and centrifugation	UPLC-FLD, excitation at 261 nm, emission at 366 nm	ChromSystems column	Isocratic mode using Chromsystems mobile phase, flow rate 1.2 mL/min	NA	0.2 μg/mL	(Jørgensen et al. 2022)
11	Isavuconazole, voriconazole, posaconazole, fluconazole, itraconazole	Human plasma	Protein precipitation using acetonitrile, vortex, and centrifugation.	UPLC-MS/MS, triple quadrupole with +ESI	C18 (50 × 2.1 mm, 1.7 μm)	Gradient mode, water, acetonitrile, formic acid, flow rate 0.4 mL/min	NA	0.2 μg/L for isavuconazole, 0.2 μg/L for voriconazole, 0.2 μg/L for posaconazole, 0.5 μg/L for fluconazole, 0.2 μg/L for itraconazole	(Toussaint et al. 2017)
12	Fluconazole, voriconazole, posaconazole, itraconazole	Human serum	Protein precipitation using acetonitrile and formic acid, vortex, and centrifugation.	UPLC-MS/MS, triple quadrupole with +ESI	C18 (30 × 2.1 mm, 1.7 μm)	Gradient mode, ammonium acetate in water, ammonium acetate in methanol and formic acid, flow rate 0.5 mL/min	0.06 μg/mL for fluconazole, 0.065 μg/mL for voriconazole, 0.029 μg/mL for posaconazole, 0.029 μg/mL for itraconazole	1 μg/mL for fluconazole, 0.1 μg/mL for voriconazole, 0.1 μg/mL for posaconazole, 0.1 μg/mL for itraconazole	(Basu et al. 2017)
13	Voriconazole	Human plasma	Protein precipitation using methanol, vortex, and centrifugation.	UPLC-MS/MS, triple quadrupole with +ESI	C18 (50 × 2.1 mm, 1.7 μm)	isocratic mode, acetonitrile: 1% formic acid (45:55), flow rate 0.50 mL/min.	NA	2 ng/mL	(Wang et al. 2015)
14	Fluconazole, posaconazole, voriconazole, itraconazole,	Human serum	Protein precipitation using diethyl ether, dichloromethane, n-hexane, and n-amyl alcohol, centrifugation.	UPLC-PDA, detection at 210–260 nm	C18 (150 × 2.1 mm, 1.7 μm)	Gradient mode, acetonitrile and ammonium bicarbonate pH 10, flow rate 0.4 mL/min	0.09 mg/L for fluconazole, 0.015 mg/L for posaconazole, voriconazole, and itraconazole,	0.3 mg/L for fluconazole, 0.05 mg/L for posaconazole, voriconazole, and itraconazole,	(Mistretta et al. 2014)

No	Analyte	Matrices	Sample Preparation	HPLC system	Stationary Phase	Mobile Phase	LOD/LLOD	LOQ/LLOQ	References
15	Voriconazole, posaconazole, isavuconazole, itraconazole	Human plasma	LLE using n-hexane and dichloromethane, centrifugation	UPLC-UV, detection at 260 nm	C6 (100 × 2.1 mm, 1.7 μm)	Gradient mode, phosphate buffer pH 2.5, acetonitrile, flow rate 0.4 mL/min	NA	0.050 μg/mL for voriconazole, 0.053 μg/mL for posaconazole, 0.054 μg/L for isavuconazole, 0.052 μg/L for itraconazole	(Verweij-van Wissen et al. 2012)
16	Posaconazole	Human plasma	protein precipitation with acetonitrile-methanol (75%/25%, vol/vol).	UPLC MS triple quad ESI	C18 (30 × 2.1 mm, 1.9 μm)	Gradient mode, ammonium formate, acetic acid in methanol, acetic acid in acetonitrile, flow rate 0.8 mL/min	NA	0.014 μg/mL	(Bertrand et al. 2010)

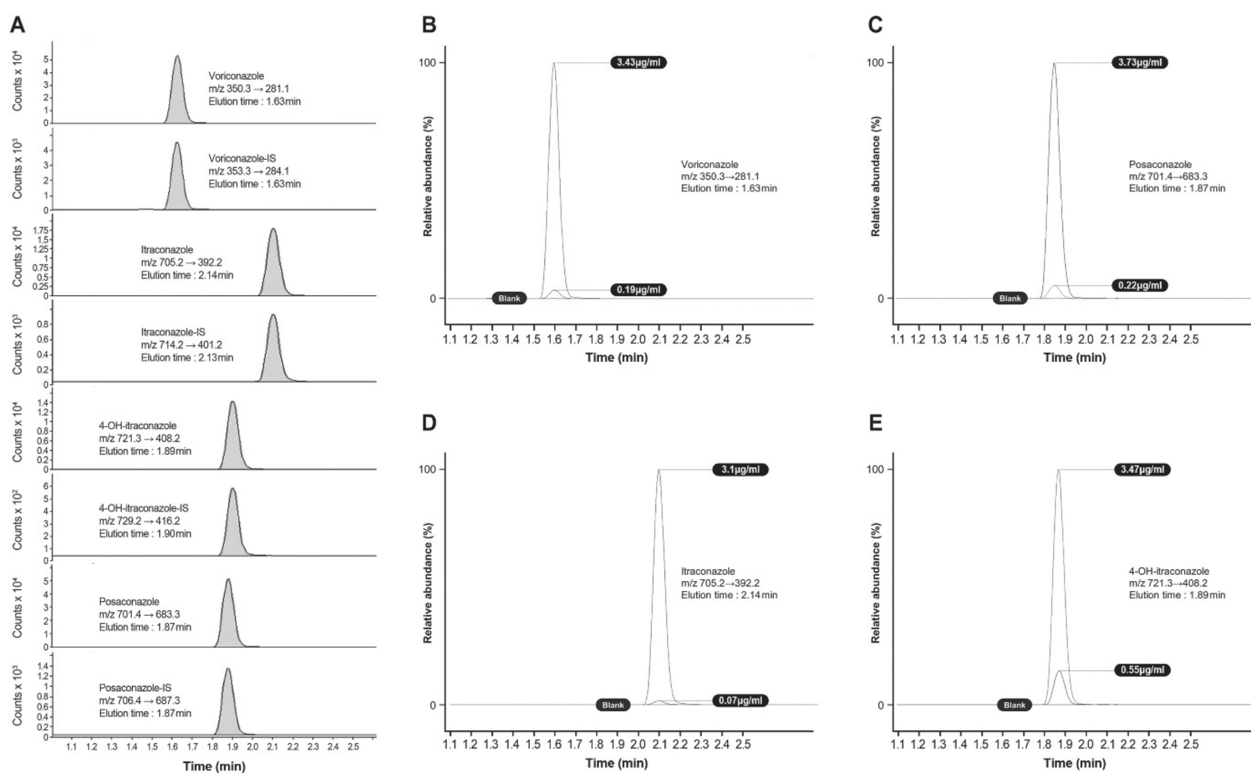

Figure 4. Chromatograms samples of blank serum (**A**) and patient samples with low and high concentrations of voriconazole (**B**), posaconazole (**C**), itraconazole (**D**), and 4-OH-itraconazole (**E**). Reuse with permission from (Yoon et al. 2019).

Microextraction technique

For the preparation of biological samples, microextraction techniques such as dispersive liquid-liquid microextraction (DLME) and solid-phase microextraction (SPME) are very helpful, especially when there is a limited number of samples to work with. The analyte and matrices properties, as well as the chromatographic and detection technique to be used must always be taken into consideration when choosing the sample preparation procedure. A method for preparing samples called solid phase microextraction combines sampling, extraction, and analyte pre-concentration into a single step. Adsorbents used in solid-phase microextraction (SPME) can take the form of a solid or a liquid, depending on the inert fiber that was coated on the polymer. Different types of analytes are transferred to the solid surface following interaction with liquid biomatrices depending on their affinity to the coated material. The fiber extracts analytes in proportion to their concentration in the sample at equilibrium (Locatelli et al. 2019). Another type of LPME is dispersive liquid-liquid microextraction. DLME is based on a ternary solvent system that utilizes a combination of water-immiscible organic solvent as the extraction solvent and water-miscible organic solvent as the disperser solvent. In DLME, a syringe is used to quickly inject a sample into an aqueous sample solution. Extraction efficiency might be increased by combining this approach with ionic liquids (ILs) or deep eutectic solvents (Manousi and Samanidou 2021). DLLME has been applied to separate triazole antifungal from the CSF sample. The extraction condition consists of a low volume of sample and solvent: 100 μL of chloroform, 100 μL of isopropyl alcohol, 200 μL of CSF, 200 μL of 50 mM phosphate buffer pH 7.3 (Zarad et al. 2021).

Solid phase extraction

Solid phase extraction (SPE) separates a mixture into desired and undesirable components in the stationary phase by using the affinity of solutes dissolved or suspended in a liquid (mobile phase). By elution with an

appropriate solvent or thermal desorption into the gas phase, analytes are recovered. This method is implemented in a number of studies as an appealing alternative to PP. SPE provides greater analyte recovery since it combines extraction and purification techniques in single or multiple steps. SPE is considered a greener technique than LLE methods since It is superior to competing methods in terms of speed, extraction efficiency, sample size, ease of automation, and compatibility with online system chromatography. The type of adsorbent used is determined by the SPE mechanism for the separation of the analyte target (Locatelli et al. 2019). Today's SPE columns provide a wide range of stationary phases, including hydrophobic phases such as C18 and C8, ion exchange, or mixed mode that can be used for optimized analyte separation in biological matrices. Most analyte extractions are performed using C18 and hydrophilic-lipophilic balance (HLB) cartridges. HLB cartridge provides some advantages over C18 because of its ability to remain saturated while simultaneously adsorbing analytes with varying polarity and pH values (Kanneti et al. 2011; Mwando et al. 2017). SPE has been widely used in the separation of triazole antifungals in biological matrices with HLB cartridges as the most common used method used in the separation (Zufía et al. 2010; Martial et al. 2018; Nannetti et al. 2018)

Molecularly imprinted polymer

Molecularly imprinted polymer (MIP) is a separation method in which polymers are synthesized using a molecular imprinting technique. This technique leaves cavities in the polymer matrix that have a certain affinity with the template molecules used. MIP has a unique affinity for certain compounds. Compared to other separation methods, MIP has several advantages: predictable structure, specific recognition of target molecules, and wide application in various fields, which makes it useful for a variety of applications, including sorbents for solid phase extraction, column chromatography stationary phase, separation of racemates, and chemical reaction catalysts. (Chen et al. 2011; Cheong et al. 2013; Arabi et al. 2020). Several methods have been used to utilize MIP for the separation of triazole antifungal agents in pharmaceutical products and biological matrices. The utilization of MIP as a novel method for sample preparation exhibits compatibility with the analysis of analytes in intricate matrices while also necessitating minimal sample volumes (Szultka et al. 2013; Manzoor et al. 2015; Zad et al. 2018).

Recommendations

The characteristics and limitations of the liquid chromatographic system, as well as the ability of detectors to detect interference from other compounds that could elute at the same retention time as the azoles, will influence the development of an analytical method for antifungal TDM. Currently, the preferred analytical technique is UPLC because of its enhanced chromatographic capabilities compared to HPLC. This preference is primarily attributed to UPLC's higher efficiency and less susceptibility to matrix effects. The type of biological sample to be analyzed (e.g., plasma, serum, cerebrospinal fluid, urine), the frequency with which determinations must be made, and the desired analytical sensitivity will determine the detector and the sample preparation method. When combined with an MS detector, it can be a powerful analytical method to analyze triazole antifungal in biological matrices.

Conclusions

Triazole antifungal drugs, commonly used for the prevention and treatment of invasive fungal infections, must be monitored by TDM to ensure successful of treatment, minimize the toxicity risk, and prevent the drug resistance. Currently, the method that has been widely used for TDM of triazole antifungal drugs in biological matrices is liquid chromatography with various detectors, which is assisted by the sample preparation method to remove sample matrices. Establishing reproducible analytical methodologies appropriate for the continuous determination of pharmaceuticals in biological samples is the first step in TDM. In order to quantify triazole antifungal medication concentrations in biological samples, chromatographic system control is necessary, such as stationary phase, mobile phase, pH, and flow rate, to get excellent results in the analytical method. Various procedures were utilized to prepare biological samples before chromatographic analysis, with protein precipitation, LLE, and SPE being the most common methods. TDM of triazole antifungal utilizing HPLC-UV usually requires extensive sample preparation and a lack of sensitivity. HPLC-FLD is rarely used as a method choice for TDM of antifungal drugs. LC-MS provided great sensitivity and selectivity, whereas extensive preparation is not required. Among all these methods, the UPLC-MS method had overcome another method due to its sensitivity and specificity, as well as its simple sample preparation and quick analysis time. Future research for TDM and analytical methods of triazole antifungal drugs should be focused on the development of selective and sensitive analytical methods and sample preparation to get a valid method that conforms to established standards.

Conflict of interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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