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Review

Advances in antifungal drug measurement by liquid chromatography-mass spectrometry

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ABSTRACT

Fungal infections, especially invasive types, have become a serious healthcare problem as the immunocompromised population increases. There are five main classes of antifungal drugs: polyenes, flucytosine, allylamines, azoles, and echinocandins. Therapeutic drug monitoring (TDM) is justified for flucytosine and triazoles due to their large inter- and intra-individual pharmacokinetic variability and their high tendency for drug-drug interactions. Available methods for measuring these drugs include bioassay, liquid chromatography and liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS). The LC-MS/MS approach is preferred due to its superior analytic sensitivity and specificity. In this review, we highlight TDM methods by LC-MS/MS for these antifungal drugs searchable in PubMed by December 1, 2018. LC-MS/MS methods that were developed for other purposes such as pharmacokinetics or toxicokinetics were also included. We have critically analyzed these methods with an emphasis on sensitivity, specificity, simplicity, throughput and robustness.

1. Introduction

1.1. Fungal infection

Fungi are eukaryotic organisms that serve as the principal decomposers in ecologic biosystems and are found abundantly worldwide. They have been used as food (such as mushroom), leavening agents and aids in fermentation process. Some species, such as *Saccharomyces cerevisiae*, are frequently used in research and in industry as a source of antibiotics and enzymes because of their fast growing and genetically modifiable properties [1].

Some fungal species can be pathogenic to humans, animals, or plants. Approximately 400 fungal species have been reported so far that can be pathogenic to humans with new species discovered every year [2]. Because fungi are abundantly present in the environment (air, soil, water, etc.), fungal infection often starts in the lung or on the skin. Unlike the molecular mechanism of bacterial infections, only limited information is available for the mechanism of fungal pathogenesis [3]. It is complex and depends on multiple factors: 1) fungal species, 2) infection sites, and 3) host immune status. The infection may be clinically unapparent or may result in diseases due to underlying cellular injuries and/or immune responses that are marked by a set of characteristic symptoms [3].

Fungal infection is divided into two classes: superficial fungal infections (SFIs) and invasive fungal infections (IFIs). SFIs affect approximately 25% of the world's population with higher rates in the undeveloped and developing countries due to their lifestyles and socioeconomic conditions [4,5]. SFIs are limited to outer layer of the body and are classified by the infection sites. Some examples in this class are tinea capitis and onychomycosis. Most IFIs are found deep inside of the solid organs and/or bloodstream in immune deficiency populations. Some commonly encountered species that cause IFIs are *Candida spp., Aspergillus spp., Cryptococcus spp.,* and others, which can be found in the environment [6,7]. IFIs are rare in general healthy population, and they are more frequently found in people with weakened immune system. The incidence of IFIs has greatly increased over the past two decades

Abbreviations: SFIs, superficial fungal infections; IFIs, invasive fungal infections; AmB, amphotericin B deoxycholate; FLZ, fluconazole; ITZ, itraconazole; VRZ, voriconazole; PSZ, posaconazole; ISZ, isavuconazole; TDM, therapeutic drug monitoring; OH-ITZ, hydroxyitraconazole; BSMM, British Society for Medical Mycology; JSC/JSTDM, Japanese Society of Chemotherapy and the Japanese Society of Therapeutic Drug Monitoring; IDSA, Infectious Diseases Society of American; HPLC, high-performance liquid chromatography; LC-MS/MS, liquid chromatography-tandem mass spectrometry; IS, internal standard; RT, retention time; LLOQ, lower limit of quantification; UPLC, ultra-high performance liquid chromatography; SPE, solid-phase extraction; LLOD, lower limit of detection; MRM, multiple reaction monitoring

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Table 1
Therapeutic ranges and toxicity levels by clinical guidelines. Table modified from [50].

Antifungal	Guideline	Time for sample draw (days)	Minimum trough concentration, efficacy (μg/mL)		Maximum trough concentration, safety (μg/mL)
			Prophylaxis	Treatment	
Flucytosine	BSMM 2014	Within 3	\	20–40	Peak < 100
	IDSA Cryptococcus 2010	3–5	\	2 h post dose > 30	Peak ≤80
Itraconazole	BSMM 2014	7	0.5	0.5	17
	IDSA Blastomycosis 2008	14	\	1	10
	IDSA Histoplasmosis 2007	14	\	1	10
Voriconazole	BSMM 2014	2–5 (ensure steady state is required)	1	1	4–6
	JSC/JSTDM 2013	5–7	\	1–2	4–5
	IDSA Histoplasmosis 2007	\	\	0.5	\
Posaconazole	BSMM 2014	7	0.7	1	\
	IDSA Histoplasmosis 2007	\	\	0.5	\

Table 2
Comparison of current assays used in antifungal drug TDM.

Assays	Equipment/ Reagent cost	Personnel requirement	Turnaround time	Sensitivity/Specificity/Selectivity	Throughput	Multiplexing
Bioassay	Low	Low	Long	Low	High	No
HPLC	Medium	Medium	Medium	Medium	Medium	Yes
LC-MS/MS	High	High	Short	High	Medium	Yes

[8,9], due to the increase of immunocompromised populations, such as those with cancer, AIDS, organ transplantation, autoimmune diseases, or under immunosuppressive therapy [10–12]. IFIs can cause serious health problems and can be fatal if untreated.

1.2. Antifungal drugs

The development of antifungal drugs has been challenging mainly due to the similarity of mammalian and fungal cells in terms of cellular structure and metabolic targets [13], which pose significant difficulty to selectively target fungi without adversely affecting host mammalian cells.

The first antifungal agent amphotericin B deoxycholate (AmB), a member of the polyene class, was introduced in the 1950s. It binds to ergosterol in the fungal membrane and causes rapid leakage of fungal cells and subsequent cell death [14,15]. This drug has broad-spectrum antifungal activity, but unfortunately it is associated with significant renal toxicity [16]. The lipid-based AmB formulations were introduced later in the 1990s with improved drug safety and toxicity. The other polyene drugs such as nystatin and pimaricinare are for topical use only. Flucytosine, which was introduced in the 1970s, inhibits both fungal DNA and RNA synthesis [17]. It is usually used in combination with other antifungal drug such as AmB to overcome drug resistance [18]. The allylamines, which includes butenafine, terbinafine, and others function by inhibiting an enzyme required for ergosterol biosynthesis [19]. The azole class includes imidazoles (clotrimazole, ketoconazole, miconazole, etc.) that are predominantly used topically and triazoles. The first generation triazoles were introduced in the 1990s and include fluconazole (FLZ) and itraconazole (ITZ). The second-generationtriazoles, voriconazole (VRZ) and posaconazole (PSZ), were launched in the 2000s with extended potency with increased activity against resistant and emerging pathogens [20]. Isavuconazole (ISZ) is the newest broad-spectrum oral and intravenous antifungal triazole which was approved by FDA in 2015 [21]. Azole antifungal agents act on inhibiting a cytochrome P450-dependnet enzyme (lanosterol 14 α-demethylase), which is essential to the biosynthesis of ergosterol in the fungal cell membrane. Long-term triazole use has been known to be problematic due to associated liver toxicity. The other drugs that are metabolized via similar pathways in the liver may result in drug-drug interactions. Lastly, the echinocandin class is the newest antifungal agents, which were introduced in the 2000s. Caspofungin, micafungin, and anidulafungin are currently approved for intravenous administration, because they are not stable in acidic conditions and therefore are not amenable for oral intake [22]. Echinocandin drugs inhibit fungal β-1,3 glucan synthase, a major enzyme complex important in the fungal cell wall synthesis. These agents have excellent activity against Candida spp. with reduced drug-drug interactions and greatly improved drug safety profiles [23].

2. Therapeutic drug monitoring

2.1. Clinical considerations

2.1.1. The needs of TDM for each antifungal class

The effective fungal infection management depends on many factors such as host's immune competency, location of the infection site, time of diagnosis, and initiation of treatment as well as antifungal drug regimen. Due to the high toxicity of most antifungal drugs, one needs to ensure that drug exposure at the site of infection is adequate while toxicity to the host is minimized. Many antifungal drugs exhibit remarkable variability in blood concentrations due to variable absorption, metabolism, elimination, and drug-drug interactions [24]. One tool that can be used to address these pharmacokinetic variability is therapeutic drug monitoring (TDM) [25]. In general, TDM is needed when the drug displays variable pharmacokinetics and has a narrow therapeutic window.

Although AmB has the longest history being used as antifungal agent, there is insufficient evidence to support routine TDM [26–29]. The three echinocandins display low oral bioavailability, while exhibit linear pharmacokinetics following intravenous administration. There is not sufficient data available to correlate their blood concentrations and therapeutic outcomes to support the routine TDM [30,31]. TDM cannot be justified for the allylamines because these drugs are for topical use.

Flucytosine has excellent bioavailability with minimum protein binding in circulation [32]. TDM is routinely used for this drug due to its significant inter-patient pharmacokinetic variability [33]. Many of the triazoles display unpredictable dose-concentration relationship, while blood concentration is predicative of treatment efficacy and drug toxicity, making these drugs good candidates for TDM. TDM of FLZ is generally not indicated because of the predictable pharmacokinetics

Table 3
Overview of methods using LC-MS/MS for AmB measurement.

Reference	Analyte	Sample preparation	IS	LC column	Mobile phase	Run time (min)	Ionization mode	MRM	Analytical range	LLOQ	Method validation	Intended use
Qin et al., [54]	AmB total	Methanol-acetonitrile protein precipitation	Paclitaxel	5 μm, 10 cm Gemini C18	0.1% formic acid aqueous; methanol-acetonitrile	7	Positive ionization; ESI	1 transition	5–2500 ng/mL	3 ng/mL	Accuracy, linearity, precision, specificity, LLOD, LLOQ, recovery, stability	In support of pre-clinical and clinical studies of an antifungal formulation of AmB
Al-Quadeib et al., [55]	AmB total	Methanol protein precipitation	Clopidogrel	1.7 μm, 10 cm BEH Shield C18	0.2% formic acid, 1% acetonitrile in 10 mM ammonium formate; 0.1% formic acid in methanol-acetonitrile	3.2	Positive ionization; ESI	1 transition	200–4000 ng/mL	200 ng/ml	Selectivity, linearity, precision, accuracy, carryover, recovery, stability	In support of pharmacokinetics of AmB in critically ill
Lee at el., [56]	Amb total and free	Methanol protein precipitation and C2 SPE	Natamycin	5 μm, 15 cm C18	Acetic acid in water and methanol	3.5 to 4	NP	1 transition	Total plasma 2–150 μg/mL; free plasma 0.001–0.20 μg/mL; total urine 0.05–30 μg/mL; total fecal homogenate 0.4–40 μg/mL	Total: 1 ng/mL; free: 2 μg/mL for plasma, 0.05 μg/mL for urine and 0.4 μg/mL for fecal homogenate	Linearity, sensitivity, selectivity, accuracy, precision, stability	In support of pharmacokinetic study of liquid-based AmB formulation

NP: not provided.

[34]. However, the inter-patient variability is greatly altered in the presence of renal dysfunction, thus FLZ TDM may be necessary [35]. ITZ is found to have large pharmacokinetic variability by different studies. The variability is related to variable metabolism of the drug due to polymorphisms of cytochrome P450 enzymes, drug-drug interaction, as well as variable absorption for different formulations [36–38]. The large variations can influence patient clinical outcomes and thus TDM is highly recommended. In addition, the metabolism of ITZ results in over 30 metabolites, one of which is hydroxyitraconazole (OH-ITZ) displaying significant antifungal activities [39]. Thus, measurement of both the original drug and the active metabolite is necessary. VRZ, one of the newer second generation triazoles, exhibits large inter-individual variations due to factors such as pharmacogenetic polymorphisms, drug-drug interaction, altered gastrointestinal absorption, inflammation, and body weight [40]. Routine monitoring of the blood level is needed. PSZ is poorly soluble in water and its absorption is intestinal pH depended [41]. Various pharmacokinetic investigations have found significant variability in both healthy and patient cohorts suggesting that TDM should be considered [42,43]. ISZ is the newest triazole and has high oral bioavailability, rapid absorption, and high protein binding in circulation. Unlike many other azoles, the pharmacokinetics of ISZ is predictable [44,45]. However, TDM could be useful in certain clinical cases when drug exposure needs to be confirmed.

2.1.2. Therapeutic and toxicity levels

Clinical guidelines are available from the British Society for Medical Mycology (BSMM) [31], the Japanese Society of Chemotherapy/the Japanese Society of Therapeutic Drug Monitoring (JSC/JSTDM) [46], and Infectious Diseases Society of America (IDSA) [47–49] regarding the therapeutic and toxicity levels of antifungal drugs (Table 1). It is worth mentioning that these concentration levels could have been determined by different methodologies. Besides these guidelines, many clinical studies may provide helpful information of the proper circulation levels of these drugs/metabolites [27].

2.2. Analytical methods

2.2.1. Bioassay

Bioassays or microbiological assays are the oldest clinical assays developed to measure blood concentrations of the antifungal drugs. Briefly, a suspension of fungi is incubated with a culture medium such as agar and is left to solidify. Clinical samples (patients' plasma/serum), calibration standards, quality controls, and blank are added individually. After incubation (at least 1 day at 37 °C), growth inhibition can be quantified by measuring the diameter of the fungi zone for each specimen. Therefore, these assays are also called diffusion assays or susceptibility assays [51].

These assays are still being used because of the relative simplicity, low cost, and commercial availability. In general, the results are comparable to other technologies used for antifungal drug measurement such as high-performance liquid chromatography (HPLC). However, these bioassays can only give overall antifungal activities, not individual drug concentrations in combined therapies. Though effort has been made to use yeast isolates that have selected susceptibilities to different antifungal drugs, selecting the proper organisms have been a challenge and the test throughput is limited [52].

2.2.2. High-performance liquid chromatography (HPLC) assays

HPLC is frequently used to measure antifungal drugs. Prior to HPLC analysis, extraction of the drugs/metabolites from blood samples is necessary. The extraction procedures can be complicated and time consuming depending on drugs of interest and desired sensitivity. The resulting samples are subject to separation on an HPLC column with signal monitored by for example an UV detector. A review of the HPLC methods for antifungal drug measurement can be found in [53].

Compared to the bioassays, the HPLC methods allow measurement

Table 4
Overview of methods using LC-MS/MS for allylamines measurement.

Reference	Analyte	Sample preparation	IS	LC column	Mobile phase	Run time (min)	Ionization mode	MRM	Analytical range	LLOQ	Method validation	Intended use
Song et al., [57]	Butenafine	n-hexane/ diethyl ether liquid-liquid extraction	Testosterone propionate	5 μm, 15 cm SymmetryShield C18	Acetonitrile and 5 mM ammonium acetate	10	NP	1 transition	0.0182–1.82 ng/ml	0.0182 ng/mL	Specificity, linearity, precision, accuracy, recovery, matrix effect, stability	Pharmacokinetic study
de Oliveira et al., [58]	Terbinafine	Diethyl ether, hexanes liquid-liquid extraction	Naftifine	4 μm, 15 cm Genesis C18	80% CH_3CN, 20% H_2O containing 10 mmol/L formic acid	5	Positive ionization; ESI	1 transition	1.0–2000 ng/mL	1.0 ng/mL	Linearity, precision, accuracy	Bioequivalence study
Dotsikas et al., [59]	Terbinafine	Methyl t-butyl ether, hexane liquid-liquid extraction	*N*-methyl-1-naphthalenemethylamine	NP	80% acetonitrile and 20% 10 mM formic acid	2.2	Positive ionization; ESI	1 transition	5.0–2000 ng/mL	NP	Accuracy, precision, recovery, stability	Bioequivalence study
Gurule et al., [60]	Terbinafine	SPE	Naftifine	5 μm, 5 cm Hypurity C18	Acetonitrile; 0.1% formic acid water	< 2	Positive ionization; APCI	1 transition	5.11–3014.19 ng/mL	5.11 ng/mL	Selectivity, linearity, precision, accuracy, recovery, matrix effect, stability	Pharmacokinetic study

NP: not provided.

Table 5
Overview of methods using LC-MS/MS for echinocandin measurement.

Reference	Analyte	Sample preparation	IS	LC column	Mobile phase	Run time (min)	Ionization mode	MRM	Analytical range	LLOQ	Method validation	Intended use
Kirchhoff et al., [61]	Caspofungin	Acetonitrile, methanol protein precipitation and online SPE	Tylosin	3.5 μm, 5 cm xTerra C8	Acetonitrile; 15 mM ammonium formate	3.5	NP	NP	0.25–40 mg/L	0.25 mg/L	Linearity, precision, specificity, ion suppression, recovery, carryover, stability	Investigate potential TDM Use
Cangemi et al., [62]	Micafungin	Acetonitrile protein precipitation	NP	1.9 μm, 5 cm Hypersil C18	5% ammonium acetate in water; acetonitrile	4	Negative ionization; ESI	5 transition	0.1–20 μg/mL	0.1 ng/mL	Ion suppression, LLOQ, linearity, precision, accuracy, recovery, stability	Clinical TDM
Boonstra et al., [63]	Micafungin	Acetonitrile, methanol protein precipitation	$^{13}C_6$-micafungin	3 μm, 10 cm Atlantis T3	20 mM ammonium formate with 5% methanol; acetonitrile	2.2	Negative ionization; ESI	NP	0.2–10 mg/L	0.1 mg/L	Selectivity, carryover, linearity, accuracy, precision, recovery, stability, dilution, matrix effect	Pharmacokinetics; TDM
van Wanrooy et al., [64]	Caspofungin and antidulafungin	Methanol protein precipitation	Aculeacin A	5 μm, 10 cm Beta basic C4	Ammonium acetate, acetic acid, trifluoroacetic anhydride water; acetonitrile, water	NP	positive ionization; ESI	1 transition	Caspofungin: 0.1–20.0 mg/L Antidulafungin: 0.5–10.0 mg/L	Caspofungin: 0.1 mg/L Antidulafungin: 0.5 mg/L	Selectivity, linearity, accuracy, precision, sensitivity, recovery, stability	Clinical TDM; research

NP: not provided.

Table 6
Overview of methods using LC-MS/MS for FLZ measurement.

Reference	Analyte	Sample preparation	IS	LC column	Mobile phase	Run time (min)	Ionization mode	MRM	Analytical range	LLOQ	Method validation	Intended use
Moraes at el., [65]	FLZ	Liquid-liquid extraction by diethyl ether/ dichloromethane	Metronidazole	4 μm, 15 cm Genesis C18	5 mM acetic acid in water and acetonitrile	4	Positive ionization; ESI	1 transition	0.05–10 μg/mL	5 ng/mL	Accuracy, precision	In support of bioequivalence of drug formulation
Wu at el., [66]	FLZ	Acetonitrile protein precipitation	D_4-FLZ	3.5 μm, 10 cm Xterra C18	0.1% formic acid in water and acetonitrile	5.4	Positive ionization; ESI	1 transition	0.01–10 μg/mL	0.01 μg/mL	Matrix effect, carryover, accuracy, precision, stability	In support of pharmacokinetic study in preterm and young term infants

of individual drugs/metabolites with improved sensitivity and specificity. The drawbacks of HPLC methods are lengthy sample preparation, long analytical time (20–30 min/sample), and relatively large sample volume required. Interference can also be a problem as compounds similar in structures may co-elute.

2.2.3. Liquid chromatography-tandem mass spectrometry (LC-MS/MS) assays

LC-MS/MS combines the separation power of HPLC and specific measurement of charged ions by MS, which offer significantly improved sensitivity and specificity. Initially LC-MS/MS is largely used by researchers and pharmaceutical companies for pharmacokinetic studies because of the instrument cost and demand on skilled operators. This technology has been increasingly used in the clinical laboratories in recent years due to the clinical needs of highly sensitive and specific methods (Table 2).

LC-MS/MS methods for measuring AmB were developed primarily for pharmacokinetic and toxicokinetic studies. Qin et al., reported an LC-MS/MS method for measuring total AmB in human and mini pig plasma in support of pre-clinical and clinical studies of one formulation of AmB. Paclitaxel is added to the samples as the internal standard (IS) followed by protein precipitation via mixing with methanol-acetonitrile. However, paclitaxel has a ~1 min retention time (RT) difference from the analyte and thus may not be ideal to compensate for matrix effects. The method has an analytical range of 5 to 2500 ng/mL [54]. Another LC-MS/MS method for measuring total AmB in plasma employs ultra-high performance liquid chromatography (UPLC) to shorten the analytical time. Similarly, sample preparation involved simple protein precipitation and the IS (clopidogrel) has a RT difference of 0.4 min from the analyte. The method has a lower limit of quantification (LLOQ) of 200 ng/mL [55]. Lee at el., reported an LC-MS/MS procedure for determination of both total and free AmB in human plasma and other biological matrices (urine and feces). Sample preparation includes protein precipitation followed by C2 solid-phase extraction (SPE). The SPE step is primarily used for analyte enrichment to increase assay sensitivity. Compared to C18 and polystyrene divinyl benzene cation exchange SPE, the authors determined that C2 SPE gave greater and more consistent analyte recovery. Free AmB separation from protein bound fractions is achieved by ultrafiltration with a molecular weight cutoff of approximately 30,000 Da. Natamycin, a structurally similar polyene, is used as the IS. Measurement of AmB in different matrices is needed to determine drug distribution for the new lipid-based formulations [56] (Table 3).

Similar to AmB, LC-MS/MS assays have been developed for allylamines that are primarily for pharmacokinetic studies. Song et al., reported a method for the measurement of butenafine in human plasma after topical administration. LC-MS/MS technique offers the needed sensitivity, since the drug concentration in plasma is relatively low. The assay has an LLOQ of 0.0182 ng/mL and is linear up to 1.82 ng/mL [57]. For terbinafine, de Oliveira et al., developed a 5 min LC-MS/MS method and applied it to bioequivalence study of two different tablet formulations. Terbinafine is extracted from human plasma by liquid-liquid extraction, dried and reconstituted for analysis. The assay has a linearity range from 1 to 2000 ng/mL [58]. Later, an automated high throughput method was reported with all liquid handling steps performed automatically by robotic workstations. The method involves a rapid sample preparation and has an LC run time of only 2.2 min with linearity range from 5 to 2000 ng/mL [59]. In 2010, Gurule et al., reduced the LC time to < 2 min in a different method. The assay employ naftifine, a structural analog of terbinafine, as the IS, which has a similar retention time as the target terbinafine. The linearity range is 5 to 3000 ng/mL [60] (Table 4).

Echinocandins are valuable to the treatment of IFIs. Kirchhoff et al., reported an LC-MS/MS method which employs protein precipitation and online SPE to speed up the work flow and to increase the robustness for caspofungin measurement. The method was developed for potential

Table 7
Overview of methods using LC-MS/MS for ITZ measurement.

Reference	Analyte	Sample preparation	IS	LC column	Mobile phase	Run time (min)	Ionization mode	MRM	Analytical range	LLOQ	Method validation	Intended use
Vogeser et al., [67]	ITZ, OH-ITZ	Acetonitrile protein precipitation and automated online SPE	Dimethyl homolog of ITZ	5 μm, 12.5 cm Li-Crospher C18	2 mM ammonium acetate acetonitrile; water, methanol	5	Positive ionization; ESI	1 transition	10–10,000 μg/L	10 μg/L	Recovery, linearity, precision, ion suppression	Clinical TDM
Kousoulos et al., [68]	ITZ, OH-ITZ	Acetonitrile and methyl t-butyl ether liquid-liquid extraction	Dimethyl homolog of ITZ	5 μm YMC-Pack ODS-A C18	80% acetonitrile and 20% 10 mM ammonium acetate	2	Positive ionization; ESI	1 transition	ITZ: 2–500 ng/mL; OH-ITZ: 4–1000 ng/mL	NP	Accuracy, precision, stability, recovery, matrix effect	Applied to a bioequivalence study
Liang et al., [69]	ITZ, OH-ITZ, ketoitraconazole and N-desalkylitraconazole	Solid-supported liquid extraction	Corresponding stable isotopic compounds	2.6 μm, 5 cm Kinetex F5	0.1% formic acid water and acetonitrile	7.6	Positive ionization; ESI	1 transition	ITZ, OH-ITZ: 5–2500 ng/mL; ketoitraconazole, N-desalkylitraconazole: 0.4–200 ng/mL	Ketoitraconazole, N-desalkylitraconazole: 0.4 ng/mL; ITZ, OH-ITZ: 5 ng/mL	Linearity, precision, accuracy, selectivity, recovery, matrix effect, stability, processed sample viability	Applied to ITZ clinical study
Suzuki et al., [70]	Total and free ITZ and OH-ITZ	SPE	D_5-ITZ and D_5-OH-ITZ	1.7 μm, 5 cm Waters Acquity BEH C18	NP	5.5	Positive ionization; ESI	1 transition	Total ITZ and OH-ITZ: 10–10,000 ng/mL; free ITZ: 0.1–20 ng/mL; free OH-ITZ 0.5–100 ng/mL	10 ng/mL for total ITZ and OH-ITZ, 0.1 ng/mL for free ITZ and 0.5 ng/mL for free OH-ITZ	Accuracy, precision, selectivity, recovery, matrix effect, stability, repeatability	Applied to TDM of patients with chronic progressive pulmonary aspergillosis and invasive pulmonary aspergillosis

NP: not provided.

Table 8
Overview of methods using LC-MS/MS for VRZ measurement.

Reference	Analyte	Sample preparation	IS	LC column	Mobile phase	Run time (min)	Ionization mode	MRM	Analytical range	LLOQ	Method validation	Intended use
Egle et al., [71]	VRZ	Automated online extraction	no IS	5 μm, 12.5 cm C18	0.1% formic acid water and acetonitrile	13 (sample prep plus LC time)	Positive ionization; ESI	3 transition	0.05–5.0 μg/mL	0.05 μg/mL	Linearity, accuracy, precision, LLOQ, specificity	Clinical TDM and pharmacokinetics
Pauwels et al., [72]	VRZ	Methanol acetonitrile protein precipitation	D_3-VRZ	C18	0.1% formic acid, 2 mM ammonium acetate water and methanol	2	Positive ionization; ESI	1 transition	0.06–20.0 mg/L	0.13 mg/L	Precision, LLOQ, selectivity, recovery, matrix effect	Clinical TDM
Keevil et al., [73]	VRZ	Acetonitrile protein precipitation	Ketoconazole	C18	0.1% formic acid, 2 mM ammonium acetate water and methanol	3	Positive ionization; ESI	1 transition	0.1–20 mg/L.	0.1 mg/L	Linearity, accuracy, precision, recovery, stability, matrix effect	Clinical TDM and pharmacokinetics
Vogeser et al., [74]	VRZ	Polypropylene protein precipitation plus automated SPE	Fenbuconazole	5 μm, 12.5 cm C18	Water, methanol; methanol, acetonitrile	4	Positive ionization; ESI	1 transition	78–5000 μg/L	NP	Ion suppression, linearity, precision	Clinical TDM
Xiong et al., [75]	VRZ	Tert-butyl methyl ether liquid-liquid extraction	Fentanyl	1.8 μm, 5 cm XDB C18	Acetonitrile 10 mM ammonium formate	1.5	Positive ionization; APCI	1 transition	50–10,000 ng/mL	50 ng/mL	Linearity, accuracy, precision	Clinical TDM
Lin et al., [76]	VRZ	Methanol protein precipitation	carbamazepine	3.5 μm, 5 cm Ultimate C18	0.1% formic acid water and acetonitrile	3	Positive ionization; ESI	1 transition	2.49–293 ng/mL	2.49 ng/mL	Specificity, linearity, accuracy, precision, recovery, matrix effect, stability	Pharmacokinetics
Wang et al., [77]	VRZ	Methanol protein precipitation	carbamazepine	1.7 μm, 5 cm BEH C18	0.1% formic acid water and acetonitrile	1	Positive ionization; ESI	1 transition	2.0–1000 ng/mL	2.0 ng/mL	Specificity, linearity, precision, accuracy, recovery, matrix effect, stability	Pharmacokinetics
Mak et al., [78]	VRZ	Protein precipitation	D_3-VRZ	2.6 μm, 10 cm kinetex C18	0.1% formic acid, 2 mM ammonium acetate water and methanol	4	Positive ionization; ESI	2 transition	0.1–10 μg/mL	0.1 μg/mL	Ion suppression, recovery, linearity, LLOQ, precision, interference, carryover, stability	Clinical TDM
Vanstraelen et al., [79]	VRZ unbound and bound	Diethylether liquid-liquid extraction	$^{13}C_2$,D_3-VRZ	2.6 μm, 5 cm Kinetex C18	Water and methanol	4.5	Positive ionization; ESI	3 transition	0.007–14.0 mg/L	0.007 mg/L	LLOQ, linearity, precision,	Pharmacokinetics
Prommas et al., [80]	VRZ	Acetonitrile protein precipitation	PSZ	2.7 μm, 5 cm Poroshell EC C18	0.1% formic acid in 10 mM ammonium acetate; acetonitrile	3.5	Positive ionization; APCI	2 transition	0.05–10 μg/ml	0.05 μg/mL	Specificity, selectivity, linearity, LLOQ, precision, accuracy, recovery, stability	Clinical TDM
Li et al., [81]	VRZ unbound and total	Acetonitrile protein precipitation	D_3-VRZ	2 μm, 5 cm C18	Water acetonitrile	3	positive ionization; ESI	1 transition	Total: 0.1–10 μg/mL, free: 0.02–2.5 μg/mL	Total: 0.007 μg/mL; free: 0.005 μg/mL	Linearity, sensitivity, matrix effect, precision, accuracy, stability	Clinical TDM

NP: not provided.

Table 9
Overview of methods using LC-MS/MS for PSZ measurement.

Reference	Analyte	Sample preparation	IS	LC column	Mobile phase	Run time (min)	Ionization mode	MRM	Analytical range	LLOQ	Method validation	Intended use
Xu et al., [82]	PSZ	Acetonitrile protein precipitation	D_4-PSZ	5 μm, 5 cm Capcell Pak C18	Acetonitrile; water and formic acid	3	Positive ionization; ESI	1 transition	2–1000 ng/mL	2 ng/mL	LLOQ, specificity, matrix effect, accuracy, precision, recovery, stability	Pharmacokinetic study
Vogeser et al., [83]	PSZ	Methanol, acetonitrile protein precipitation followed by on-line SPE	PSZ related compounds	5 μm, 7.7 cm Li Chrospher C18	Water/methanol; 0.1% formic acid methanol	4	Positive ionization; ESI	1 transition	250–4190 μg/L	NP	Recovery, specificity, reproducibility, accuracy, linearity, sensitivity, carryover, stability	Clinical TDM
Kruger et al., [84]	PSZ	Methanol protein precipitation	PSZ related analogue	1.7 μm, 5 cm BEH C18	0.1% formic acid and 2 mM ammonium formate water; methanol	2.6	Positive ionization; ESI	1 transition	25–3634 μg/L	37 μg/L	Precision, recovery, LLOD, LLOQ, linearity	Clinical TDM

NP: not provided.

TDM use. Tylosin, a veterinary drug, is used as the IS. The analytical measurement range is from 0.25 to 40 mg/L [61]. Though the relationship between micafungin blood level and treatment outcome is currently undefined, Cangemi et al., developed an LC-MS/MS method measuring micafungin and applied it to drug dose adjustment in infants. The assay has an LLOQ of 0.1 ng/mL with an overall turnaround time of 20 min [62]. Another method by Boonstra et al., employs isotopic labelled micafungin IS and a simple protein precipitation as the sample preparation step. The assay has a linearity range of 0.2 to 10 mg/L [63]. Simultaneous measurement of caspofungin and antidulafungin in plasma is achieved by using aculeacin A as IS for both analytes. Caspofungin has an analytical measurement range of 0.1 to 20.0 mg/L and antidulafungin 0.5 to 10.0 mg/L [64] (Table 5).

There are plenty LC-MS/MS methods available in literature for measuring triazoles. An LC-MS/MS method was reported to measure FLZ concentration in plasma utilizing metronidazole as IS [65]. Plasma sample is extracted with diethyl ether/dichloromethane (70/30). The resulting organic layer containing the analyte is removed, dried and reconstituted prior to LC-MS/MS analysis. The assay is linear from 0.05 to 10 μg/mL. Though liquid-liquid extraction is more labor intense, the cost is generally lower compared to SPE. However, the analyte and IS are not co-eluted with ~0.3 min difference in RT. Protein precipitation for sample preparation and a stable isotopic labelled compound as IS, which co-elutes with the analyte, are used in another LC-MS/MS method [66]. Stable isotopic labelled IS may be better to correct for matrix effects. The assay only requires 70 μL of plasma, which is suitable for patient populations who cannot offer much blood for testing. The assay has a linear range of 0.01 to 10 μg/mL [66] (Table 6).

In 2003, Vogeser et al., reported an LC-MS/MS method for the simultaneous determination of both ITZ and its active metabolite OH-ITZ with automated online SPE using a dimethyl homolog of itraconazole as the IS for both analytes [67]. Typically, concentration of the active metabolite OH-ITZ is higher in circulation than ITZ, and measurement of both is needed for accurate assessment of antifungal treatment effects. Another high throughput method was published two years later which utilizes liquid-liquid extraction for sample preparation and a dimethyl homolog of ITZ as the IS [68]. All liquid transfer steps, including preparation of calibrators and quality controls as well as IS addition, are performed automatically using a robotic liquid handling workstation. The method is sensitive and specific for both ITZ and OH-ITZ. It also demonstrates an excellent linear range of 2 to 500 ng/mL for ITZ and 4 to 1000 ng/mL for OH-ITZ. In a more recent report, Liang et al., developed and validated an LC-MS/MS assay for simultaneous determination of ITZ and its three metabolites, OH-ITZ, ketoitraconazole and N-desalkylitraconazole. Human plasma is extracted using a solid supported liquid extraction technique and each IS used is a corresponding stable isotopic labelled compound. The linearity range for ITZ and OH-ITZ is up to 2500 ng/mL, and for ketoitraconazole and N-desalkylitraconazole the linearity range is 0.4 to 200 ng/mL [69]. Later on, an UPLC-MS/MS method quantifying both total and free ITZ and OH-ITZ was reported. Plasma samples are ultra-filtrated for the measurement of free ITZ and OH-ITZ. SPE is used as an enrichment step prior to MS analysis for both total and free analyte measurement. The LLOQ is 10 ng/mL for total ITZ and OH-ITZ, 0.1 ng/mL for free ITZ and 0.5 ng/mL for free OH-ITZ. This method was applied to TDM of patients with chronic progressive pulmonary aspergillosis and invasive pulmonary aspergillosis. Plasma free OH-ITZ concentrations were multiple folds higher than plasma free ITZ concentrations in the three patients tested [70] (Table 7).

There are many methods of VRZ measurement by LC-MS/MS in the literature. These methods differ by the amount of sample used (the minimum is 5 μL and the maximum is 200 μL); sample preparation (protein precipitation, liquid-liquid extraction, SPE, and online extraction); LC analytical time (from 1 min to over 10 min including online extraction); LLOQ (from 0.002 μg/mL to 0.1 μg/mL) [71–81]. Only four of these methods employed isotopic labelled VRZ as IS [72,78,79,81],

Table 10
Overview of methods using LC-MS/MS for multiple triazole measurement.

Reference	Analytes	Sample preparation	IS	LC column	Mobile phase	Run time (min)	Ionization mode	MRM	Analytical range	LLOQ	Method validation	Intended use
Alffenaar et al., [85]	VRZ, FLZ, ITZ, OH-ITZ and PSZ	Methanol and acetonitrile protein precipitation	Cyanoimipramine	5 μm, 5 cm C18	Ammonium acetate, acetic acid, trifluoroacetic anhydride water and acetonitrile	3.6	positive ionization; ESI	1 transition	FLZ: 0.5–200 mg/L VRZ: 0.05–10 mg/L ITZ: 0.1–5 mg/L OH-ITZ: 0.1–5 mg/L PSZ: 0.1–10 mg/L	FLZ: 0.5 mg/L VRZ: 0.1 mg/L ITZ: 0.1 mg/L OH-ITZ: 0.1 mg/L PSZ: 0.1 mg/L	Selectivity, linearity, accuracy, precision, recovery, stability	Clinical TDM
Beste et al., [86]	VRZ, FLZ, ITZ and PSZ	Methanol protein precipitation and online SPE	Ketoconazole	5 μm, 5 cm Allure PFP Propyl	2 mM ammonium formiate acetonitrile and water	3	Positive ionization; ESI	2 transition	14–10,000 ng/ml	14 ng/mL	Specificity, linearity, precision, accuracy, recovery, carryover, stability	Clinical TDM
Couchman et al., [87]	VRZ, FLZ, ITZ, PSZ and OH-ITZ	Online turbo flow sample extraction	Ketoconazole and ITZ analogue	Gemini Phenyl C6	Methanol/water	12	Positive ionization; APCI	2 transition	ITZ, OH-ITZ, PSZ: 0.05–5.0 mg/L VRZ, FLZ: 0.1–10 mg/L	NP	Accuracy, precision, recovery, ion suppression, LLOD	Clinical TDM
Jenkins et al., [88]	ITZ, OH-ITZ, VRZ and PSZ	Acetonitrile protein precipitation	Deuterium labelled IS for each analyte	BEH C18	2 mM ammonium acetate with 0.1% formic acid in water and methanol.	5	Positive ionization; ESI	2 transition	VRZ: 0.13–6.54 mg/L PSZ: 0.16–5.66 mg/L ITZ: 0.18–3.64 mg/L OH-ITZ: 0.18–3.81 mg/L	0.1 mg/L	Linearity, LLOD, LLOQ, precision, accuracy, ion suppression, ion ratio, method comparison	Clinical TDM
Muller et al., [89]	FLZ, ISZ, ITZ, OH-ITZ, PSZ, VRZ	Acetonitrile protein precipitation	Deuterium labelled IS for each analyte	1.9 μm, 5 cm Hyersil gold C18	0.1% formic acid/ acetonitrile	3	Positive ionization; ESI	2 transition	FLZ: 0.5–40 mg/L ISZ: 0.1–9 mg/L ITZ: 0.1–4 mg/L OH-ITZ: 0.05–4 mg/L PSZ: 0.05–8 mg/L VRZ: 0.1–6 mg/L	FLZ: 28.3 μg/L ISZ: 1.0 μg/L ITZ: 1.7 μg/L OH-ITZ: 26.2 μg/L PSZ: 10.3 μg/L VRZ: 6.3 μg/L	Matrix comparison, linearity, accuracy, precision, LLOD, LLOQ, carryover, stability, recovery, matrix effect	Clinical TDM
McShane et al., [90]	FLZ, ISZ, ITZ, OH-ITZ, PSZ, VRZ	Acetonitrile protein precipitation	Deuterium labelled IS for each analyte	2.6 μm, 5 cm Accucore RP	0.1% formic acid in water and acetonitrile	2	Positive ionization; ESI	2 transition	FLZ: 0.5–30 μg/mL all others: 0.2–10 μg/mL	FLZ: 0.5 μg/mL all others: 0.2 μg/mL	Ion suppression, mixing study, interference, analytical measuring range, carryover, stability, precision, method comparison	Clinical TDM
Xiong et al., [92]	VRZ, ITZ, OH-ITZ	Tertbutyl methyl ether liquid-liquid extraction	Loratadine	1.8 μm, 5 cm Zorbax	10 mM ammonium formate acetonitrile and water	4.5	Positive ionization; APCI	1 transition	VRZ: 0.05–10 μg/mL	VRZ: 0.05 μg/mL	Selectivity, linearity, accuracy, precision,	Clinical TDM

(*continued on next page*)

Table 10 (*continued*)

Reference	Analytes	Sample preparation	IS	LC column	Mobile phase	Run time (min)	Ionization mode	MRM	Analytical range	LLOQ	Method validation	Intended use
				Eclipse XDB-C18					ITZ, OH-ITZ: 0.025–5 μg/mL	ITZ, OH-ITZ: 0.025 μg/mL	recovery, matrix effect, stability	
Verdier et al., [93]	FLZ, ITZ, PSZ, VRZ, OH-ITZ	Acetonitrile protein precipitation	Dimethyl-ITZ for all analyte	3 μm, 5 cm C18 Hypersil Gold	0.1% formic acid, water and acetonitrile	15	Positive ionization; ESI	1 transition	0.1–12 μg/mL	0.1 μg/mL	Specificity, selectivity, precision, accuracy, recovery, matrix effect, stability	Clinical TDM
Baietto et al., [91]	ITZ, PSZ, VRZ	n-hexane/ethyl acetate liquid-liquid extraction	Dimethyl diquinoxaline	5 μm, 15 cm C18	0.05% formic acid in water and acetonitrile	NP	Positive ionization; ESI	1 transition	ITZ, PSZ: 0.031–8 mg/L VRZ: 0.058–15 mg/L	ITZ, PSZ: 0.031 mg/L VRZ: 0.058 mg/L	Accuracy, precision, recovery, matrix effect, stability, specificity, LLOD, LLOQ	Clinical TDM

NP: not provided.

which is recommended for matrix effect compensation. In addition, less than half of the methods monitor two or more multiple reaction monitoring (MRM) transitions for the analyte, which is also highly recommended for improving identification confidence. One method is able to measure both total and unbound VRZ in plasma. It is believed that the unbound VRZ is a better predictor of therapeutic efficacy especially for cancer patients with hypoalbuminemia [81]. Saliva is shown to be a reliable alternative specimen type for VRZ TDM by LC-MS/MS. R values for saliva versus unbound and total plasma VRZ concentrations are 0.970 ($p < 0.001$) and 0.891 ($p < 0.001$), respectively. In this study the unbound VRZ is separated from protein bound fractions by equilibrium dialysis [79] (Table 8).

For PSZ, Xu et al., reported a method that uses acetonitrile protein precipitation for sample preparation and d_4-PSZ as the IS. The method is linear from 2 to 1000 ng/mL [82]. A method by Vogeser et al., employs on-line SPE to reduce manual sample handling with an LC analytical time of 4 min [83]. Another method utilizes UPLC to shorten LC analytical time [84]. However, all of these methods monitor only one MRM transition for each analyte and IS, which is not ideal for analyte identification (Table 9).

Beside assays for the measurement of a single triazole antifungal drug, more and more methods become available in literature to measure multiple triazoles simultaneously. Alffenaar et al., reported a method measuring VRZ, FLZ, ITZ, OH-ITZ and PSZ in human serum by LC-MS/MS after protein precipitation using a single IS cyanoimipramine [85]. In a similar method by Beste et al., online SPE is introduced after protein precipitation and ketoconazole is used as the IS for all 4 analytes in plasma. The advantage of this assay is that it is rapid with only 3 min run time between injections [86]. Couchman et al., analyzed VRZ, FLZ, ITZ, PSZ and OH-ITZ in plasma and serum by online automated turbo-flow sample extraction followed by LC-MS/MS analysis to reduce manual sample preparation [87]. In another report, ITZ, OH-ITZ, VRZ and PSZ are analyzed in an UPLC-MS/MS method with < 5 min LC analytical time between injections. A deuterium labelled IS is used for each analyte [88]. Muller et al., reported a 3-min method that measures 5 triazoles (FLZ, ISZ, ITZ, PSZ and VRZ) and 1 active metabolite (OH-ITZ) after protein precipitation. An isotopic labelled compound is used as IS for every analyte. The assay only requires 50 μL of plasma sample, and the LLOQ ranges from 1.0 μg/mL (ISZ) to 28.3 μg/mL (PLZ) [89]. We also developed a fast (2 min) LC-MS/MS method with dilute and shoot approach after protein precipitation for analyzing FLZ, ISZ, ITZ, PSZ, VRZ and OH-ITZ. It requires only 50 μL of plasma or serum. Isotopic labelled IS is used for each and every analyte. Ion suppression is observed for FLZ as it elutes early on the LC chromatogram; however, this matrix effect is corrected by the IS. This demonstrates the importance of using isotopic labelled IS because of its ability to compensate for matrix effects. The linear range is from 0.2 to 10 μg/mL for all analytes except FLZ, for which the linear range is 0.5 to 30 μg/mL [90]. Besides blood, dried plasma spot as the sample type for triazole measurement has been explored as a cost effective strategy in sample handling. Three triazoles (ITZ, PSZ, and VRZ) are measured by the developed LC-MS/MS method. A high degree of correlation ($R^2 > 0.94$) is obtained between the dried plasma spot method and the standard (blood plasma) method [91]. Two additional methods of measuring multiple triazoles [92,93] are also included in Table 10.

Finally, there are methods in the literature that measure triazoles and other antifungal drugs simultaneously. Farowski et al., described a method that measures 3 triazoles (ISZ, PSZ and VRZ) and 3 echinocandins (anidulafungin, caspofungin and micafungin) in an 8 min run by LC-MS/MS. However, corresponding isotopic labelled IS are not used for all analytes [94]. Decosterd et al., reported a method to measure 4 triazoles (FLZ, ITZ, PSZ and VRZ), 2 metabolites (OH-ITZ and voriconazole-N-oxide), and 2 echinocandins (aniduafungin and caspofungin) in a 7 min run by UPLC-MS/MS. Corresponding deuterated compounds of triazoles are used as IS for all analytes [95]. Furthermore, Jourdil et al., expanded the list to 11 drugs belonging to disparate

Table 11
Overview of methods using LC-MS/MS for multiple antifungal drug class measurement.

Reference	Analytes	Sample preparation	IS	LC column	Mobile phase	Run time (min)	Ionizationmode	MRM	Analytical range	LLOQ	Method validation	Intended use
Farowski et al., [94]	ISZ, PSZ and VRZ; anidulafungin, caspofungin and micafungin	Density gradient and acetonitrile protein precipitation	Echinocandins and ISZ derivate	10 cm Beta Basic C4	Water and methanol	8	Positive ionization; ESI	1 transition	Anidulafungin: 64–6400 ng/mL caspofungin: 108–5400 ng/mL micafungin: 160–8000 ng/mL ISZ: 12.5–5000 ng/mL PSZ: 15.5–6200 ng/mL VRZ: 12–4800 ng/mL	Anidulafungin: 64 ng/mL caspofungin: 108 ng/mL micafungin: 160 ng/mL ISC: 4.5 ng/mL PSZ: 10 ng/mL VRZ: 4.2 ng/mL	Linearity, accuracy, precision, LLOD, LLOQ, stability, recovery	Pharmacokinetics peripheral blood compartment analysis
Decosterd et al., [95]	FLZ, ITZ, PSZ and VRZ; OH-ITZ and voriconazole-N-oxide; aniduafungin and caspofungin	Acetonitrile protein precipitation	Deuterated compounds of triazoles	1.7 μm, 3 cm Acquity C18	0.1% formic acid in water and acetonitrile	7	Positive ionization; ESI	1 to 3 transitions	FLZ: 0.1–50 μg/mL voriconazole-N-oxide: 0.01–5 μg/mL VRZ: 0.02–10 μg/mL caspofungin: 0.06–30 μg/mL OH-ITZ: 0.02–10 μg/mL PSZ: 0.02–10 μg/mL aniduafungin: 0.1–12 μg/mL ITZ: 0.02–10 μg/mL	FLZ: 0.1 μg/mL voriconazole-N-oxide: 0.01 μg/mL VRZ: 0.02 μg/mL caspofungin: 0.06 μg/mL OH-ITZ: 0.02 μg/mL PSZ: 0.02 μg/mL aniduafungin: 0.1 μg/mL ITZ: 0.02 μg/mL	LLOQ, accuracy, precision, stability, matrix effect, recovery, selectivity	Clinical TDM
Jourdil et al., [96]	PSZ, VRZ, ITZ, OH-ITZ	Acetonitrile protein precipitation plus online sample clean up	Shared deuterated compounds	2.6 μm, 5 cm pentafluorophenyl	10 mM ammonium formate water, 0.1% formic acid acetonitrile	4	Positive ionization; ESI	2 transition	PSZ: 0.1–15 mg/L VRZ: 0.1–15 mg/L ITZ: 0.1–15 mg/L OH-ITZ: 0.1–15 mg/L	PSZ: 0.1 mg/L VRZ: 0.1 mg/L ITZ: 0.1 mg/L OH-ITZ: 0.1 mg/L	Selectivity, linearity, accuracy, precision, recovery, matric effect, stability	Clinical TDM
Qu et al., [97]	AmB, FLZ, and flucytosine	Online SPE	Carbamazepine, 5-methylcytosine hydrochloride	5 μm 2 cm	Acetonitrile 10 mM ammonium formate buffer	7	Positive ionization; ESI	Targeted selected ion monitoring	AmB: 0.04–2.00 μg/mL FLZ: 0.04–2.0 μg/mL flucytosine: 0.4–20 μg/mL	0.04 μg/mL for all	Linearity, LLOQ, accuracy, precision, recovery, matrix effect, stability	Clinical TDM

pharmacological classes: antifungals (azoles), antibiotics, antineoplastic and antiretroviral agents for simultaneous quantification on a 2-dimensional LC-MS/MS assay which includes a sample clean up column in front of the analytical column. The total LC time is only 4 min. Protein precipitation of 10 μL plasma with acetonitrile is used as the only sample preparation step. However, corresponding IS are not used for all analytes [96]. Lastly, an interesting study was published in 2017 on the development of an automated online-dual-SPE-LC coupled with high resolution MS method for determination of AmB, FLZ, and flucytosine in human plasma and CSF. The quantification mode was target-selected ion monitoring [97] (Table 11).

3. Conclusion

Measurement of antifungal drugs can be achieved by bioassays, HPLC, or LC-MS/MS. TDM is justified for flucytosine and triazoles due to their large inter-individual and intra-individual pharmacokinetic variability and their high tendency of drug-drug interaction. For others, methods are developed primarily for pharmacokinetic and toxicokinetics studies. Among all these methods, LC-MS/MS assays have demonstrated superior sensitivity and specificity with simple sample preparation and short analytical time.

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