JMB 2009; 28 (1) DOI: 10.2478/v10011-008-0040-1

UDK 577.1 : 61 **ISSN 1820-7367**

JMB 28: 1–10, 2009

Review article
Pregledni članak

ANTIFUNGAL AZOLE METABOLITES: SIGNIFICANCE IN PHARMACEUTICAL AND BIOMEDICAL ANALYSIS

METABOLITI ANTIFUNGICIDNOG AZOLA: ZNAČAJ U FARMACEUTSKIM I BIOMEDICINSKIM ANALIZAMA

Sachin Kokil, Manish Bhatia

Department of Pharmaceutical Chemistry, Bharati Vidyapeeth College of Pharmacy, Kolhapur Maharashtra, India (416013)

Summary: Individualised therapy and factors determining such variability among patients are confusing to both physicians and their patients because of the observed therapeutic, metabolic and toxic response. The same is true about antifungal azoles. They are under the influence and become targets of metabolic drug-drug interactions where more than one active form of the drug may be involved. The clinical relevance of these interactions may vary upon the azole involved and upon the intention of drug administration. The pharmacodynamics and pharmacokinetics of azole drugs as indicated by the reviewed data make the need for characterization of all their metabolites even more evident. The health care systems also emphasize the identification and quantitation of the metabolites for a comprehensive understanding of the biological safety of individual metabolites, thus, revealing the need and scope of bioanalytical research in metabolite and toxicity profiling of drugs. Availability of protocols for qualitative and quantitative characterization of all metabolites will have many applications for therapeutic drug monitoring, bioequivalence, toxicological and all related studies. Identification of metabolites may be done by a variety of chromatographic and spectroscopic techniques, either alone or in combination with other techniques. Conventional liquid chromatography has been exploited widely in the field of metabolite profiling. The arrival of hyphenated techniques has revolutionized metabolite profiling, by not only separating but also generating data for the structural identification of

Kratak sadržaj: Individualizovana terapija i faktori koji određuju varijabilnost među pacijentima zbunjuju i lekare i pacijente zbog očiglednog terapeutskog, metaboličkog i toksičnog odgovora. Isto važi i za antifungicidne azole. Oni su pod uticajem, i postaju mete metaboličkih interakcija između lekova, koje mogu obuhvatiti više od jedne aktivne forme leka. Klinički značaj tih interakcija se razlikuje u zavisnosti od azola i cilja primene leka. Farmakodinamika i farmakokinetika azola, prema dosadašnjim podacima, dodatno ističu potrebu za karakterizacijom svih metabolita azola. Sistemi zdravstvene zaštite takođe naglašavaju identifikaciju i kvantifikaciju metabolita u cilju razumevanja biološke bezbednosti pojedinih metabolita, otkrivajući na taj način potrebu i obim bioanalitičkog istraživanja u profilisanju metabolita i toksičnosti lekova. Dostupnost protokola za kvalitativnu i kvantitativnu karakterizaciju svih metabolita omogućiće široku primenu u terapijskom praćenju leka, bioekvivalenciji, toksikološkim i svim srodnim studijama. Identifikacija metabolita može se postići pomoću različitih hromatografskih i spektroskopskih tehnika, pojedinačno, ili u kombinaciji sa drugim tehnikama. Uobičajena tečna hromatografija često se primenjuje u profilisanju metabolita. Podeona tehnika je donela revoluciju u profilisanju metabolita, na taj način što je, pored razdvajanja, pružila i podatke za strukturnu identifikaciju metabolita. Od svih tehnika najviše se koriste tečna hromatografija-masena spektroskopija, spektroskopija nuklearne magnetske rezonancije, tečna hromatografija-spektroskopija nuklearne magnetske rezonancije, tečna

Address for correspondence:

Dr. Manish Bhatia
Professor and Head,
Department of Pharmaceutical Chemistry
Bharati Vidyapeeth College of Pharmacy
Near Chitranagri, Morewadi
Kolhapur, Maharashtra, India. Pin:-416013
Phone: +919822172940; Fax: +912312638392
e-mail: bhatiamanish13@gmail.com

List of Abbreviations: CYP, cytochrome; FDA, food and drug administration; GC-MS, gas chromatography-mass spectrometry; HPLC, high performance liquid chromatography; IR, infrared; ITZ, itraconazole; LC-DAD, liquid chromatography with diode array detector; LC-MS, liquid chromatography-mass spectrometry; LC-NMR, liquid chromatography-nuclear magnetic resonance; LC-NMR-MS, liquid chromatography-nuclear magnetic resonance-mass spectrometry; LLE, liquid-liquid extraction; MS, mass spectrometry; ND-ITZ, N-desalkyl itraconazole; NMR, nuclear magnetic resonance spectroscopy; OH-ITZ, hydroxy itraconazole; P-gp, P-glycoprotein; SPE, solid phase extraction; SPE-GC, solid-phase extraction-gas chromatography; SPME-LC, solid-phase microextraction-liquid chromatography; UV, ultraviolet.

metabolites as well. Among all techniques, the most exploited are Liquid Chromatography-Mass Spectroscopy, Nuclear Magnetic Resonance spectroscopy, Liquid Chromatography-Nuclear Magnetic Resonance spectroscopy, Liquid Chromatography-Nuclear Magnetic Resonance spectroscopy-Mass Spectroscopy and Extraction-Nuclear Magnetic Resonance spectroscopy. This compilation provides a tool for the metabolic, bioanalytical and biomedical understanding of antifungal azole metabolites.

Keywords: azole antifungals, bioanalytical, biomedical analysis, chromatography, metabolites, spectrophotometry

hromatografija-spektroskopija nuklearne magnetske rezonancije-masena spektroskopija, i ekstraciona spektroskopija nuklearne magnetske rezonancije. Ova kompilacija može doprineti metaboličkom, bioanalitičkom i biomedicinskom razumevanju antifungicidnih metabolita azola.

Ključne reči: antifungicidi azola, bioanalitička analiza, biomedicinska analiza, hromatografija, metaboliti, spektrofotometrija

Introduction

An azole is a class of five member nitrogen heterocyclic ring compounds containing at least one other noncarbon atom, nitrogen, sulphur or oxygen (1). The azole includes pyrrole, pyrazole, imidazole, triazole, tetrazole, oxazole, isoxazole, thiazole, isothiazole. The azoles, as a group of antifungal drugs, act by inhibiting the C14 demethylation of sterols. This is a key step in the conversion of lanosterol to ergosterol. The demethylation is a multistep, enzyme mediated pathway involving a series of oxidation steps catalysed by the iron haem protein cytochrome P450. This is a site normally occupied by O_2, and is a key part of the activation of molecular oxygen for the oxidation process that the enzyme mediates. Inhibitions of these steps lead to a build up of C14 methyl sterols, which replace ergosterol in the cell membrane. This results in a series of changes in the fungal cell, e.g. change in cell membrane; an increase in cell volume; abnormalities in cell division and cell function. This suggests that the antifungal activity of azoles is based on the inhibition of sterol biosynthesis, particularly catalyzed by lanosterol 14 demethylase (CYP51), a widely distributed and highly conserved cytochrome P450 dependent monooxygenase (2–5).

Unlike bacteria, both fungi and humans are eukaryotes. Thus fungal and human cells are similar at the molecular level. Therefore it is more difficult to find a weakness in the fungi to attack that does not also exist in human cells, and so if you attack the fungus, you may also attack the human cells. Subsequently there are often side effects to some of azole antifugals and the major limitation of the azole antifungal drugs is the frequency of their interactions with co-administered drugs, which result in adverse clinical consequences (6). One type of azole drug interaction may lead to decreased plasma concentration of the azole, related to either decreased absorption or increased metabolism of the azole. A second type of azole drug interaction may lead to an unexpected toxicity of the co-administered drug, relating to the ability of the azoles to increase plasma concentrations of other drugs by altering hepatic metabolism via the cytochrome P450 system. Another constraint for azole antifungals is, ethnic differences are puzzling to physicians because of the sensible toxic, metabolic and therapeutic response. Identification of these sensible effects in terms with newly formed entities like metabolites is important to design new drug candidates with improved pharmacological profiles.

Consideration of structures of metabolites is functional both for optimizing the metabolic stability of a drug as well as rationalizing the drug safety profile. Protocols for qualitative and quantitative description of azole metabolites will have numerous applications for therapeutic drug monitoring, bioequivalence, toxicological and all related studies. Multifaceted study of these proceedings is required in the case of azole metabolites. Present publication reviews the metabolic, bioanalytical and biomedical comprehensions of antifungal azole metabolites.

Azole antifungal agents

The availability over the past two decades of the azole antifungal agents represents a major advance in the management of systemic fungal infections.

There are two groups in clinical use:

1. Imidazoles (e.g. ketoconazole, miconazole, clotrimazole, and econazole)
2. Triazoles (e.g. fluconazole, itraconazole, voriconazole, and posaconazole).

Miconazole, the first azole drug to be approved and now recently withdrawn from the market, was available only as a highly toxic i.v. formulation; consequently, it was only rarely used. By contrast, the three oral azoles, ketoconazole, imidazole, and, especially, itraconazole and fluconazole (both triazoles), have become frequently used therapeutic alternatives to amphotericin B (7, 8). The relatively broad spectrum of activity of the azoles against common fungal pathogens (e.g., *candida species*, *cryptococcus neoformans*, *blastomyces dermatitidis*, *histoplasma capsulatum*, *coccidioides immitis*, *paracoccidioides brasiliensis*, *sporothrix schenckii*, and *aspergillus species* (only itraconazole is active), ease of administration, and limited toxicity are highly attractive features.

Among the oral azoles, fluconazole (also available as an i.v. formulation) possesses the most desirable pharmacologic properties, including high bioavailability, high water solubility, low degree of protein binding, wide volume of distribution into body tissues and fluids, including cerebrospinal fluid, and urine, and long half-life (9–11). In addition, fluconazole and itraconazole are better tolerated and more effective than ketoconazole. As the triazoles have greater affinity for fungal compared with mammalian P450 enzymes, their safety profile is significantly improved over the imidazoles. The triazoles have broad applications in therapy (12–18).

Imidazoles

First generation imidazoles

They have exceptional skin/mucous membrane compatibility and so are useful for topical application. Competition to clotrimazole and miconazole, came in the form of econazole nitrate. Miconazole provides an exception to the others in that it is absorbed in the gut slightly better. It binds strongly to lipoproteins: up to 95% is protein bound and only 20–30% of a dose is detectable in the blood. Its effective concentration is therefore much reduced. Additionally, the imidazole ring is subject to considerable metabolic inactivation.

Second generation imidazoles

Improvement upon miconazole and econazole, but they retain the important advantages of the early imidazoles, namely high antimycotic activity and abroad spectrum of activity. The developed molecule was ketoconazole.

The imidazole ring was identified as the main problem with regards to the metabolic vulnerability; of all the hetero cycles tested, the 1,2,4-triazole ring was the only one with which activity was retained. In comparison to the imidazoles, the triazolyl compounds showed reduced activity *in vitro*, but increased activity *in vivo*. This is because the triazole ring was far less readily metabolized. The bis-triazolyl compounds were the breakthrough point. The 2,4-dichlorophenyl bis-triazolyl candidate was stable *in vivo*. All aryl-bis--triazolyl variations gave active compounds, but only the 2,4-difluoro was not toxic.

Triazoles

First generation triazoles

Fluconazole has good overall activity against *Candida species* and *Cryptococcus neoformans*. However, resistance to the drug is encountered in certain non-albicans *Candida species* such as *C. krusei* and some isolates of *C. glabrata* (19). It is available as oral and intravenous formulations. Itraconazole has activity against yeasts and some moulds (including *Aspergillus*), but is disadvantaged by variable bioavailability and an unpleasant taste. The bioavailability of the capsule form is highly influenced by concomitant food intake, and there is considerable intra and inter patient variability in plasma drug concentrations. The solution form has a more favourable pharmacokinetic profile (20–21).

Second generation triazole agents

Voriconazole and posaconazole are second generation triazoles with an extended spectrum of activity against yeasts, *C. neoformans* and moulds, including *Aspergillus*, *Scedosporium* and *Fusarium species*. Voriconazole is active against fluconazole resistant *Candida species*, although cross-resistance has been observed. Posaconazole is the broadest spectrum azole to date. Posaconazole is the only azole with clinical activity against zygomycete fungi. Its absorption is improved when taken with food or nutritional supplements.

Azoles in the treatment of human diseases

In the treatment of systemic and dermal mycoses, azoles play an essential role (22). They show significantly fewer side-effects in comparison with other antimycotics such as amphotericin B, they can be applied after the emergence of resistance to other antimycotics and they are inexpensive (23–24). Another significant application of azoles is the management of advanced estrogen responsive breast tumours in postmenopausal women (25–27). This local production can substantially support the growth of estrogen responsive tumours, and treatment with azole compounds (letrozole, anastrozole, vorozole, or fadrozole) suppresses estrogen production dramatically in these patients. Consequently, tumour growth can be blocked and sometimes even remission occurs (28–31). Currently the two triazoles, letrozole and anastrozole, are in use, partially replacing the classical nonsteroidal antiestrogen tamoxifen. In a clinical trial, anastrozole was also effective in the treatment of estrogen dependent endometriosis (32). Furthermore, letrozole was used to treat boys with delayed puberty and short stature to induce growth (33). The rationale behind this treatment is that azoles inhibit estrogen dependent bone maturation. This leads to a delayed closure of the epiphysial growth plates and thereby allows growth. These examples demonstrate the increasing importance of azole compounds in sex steroid hormone dependent diseases.

Drug-drug interactions are a major concern with triazoles. Azoles are potent metabolic inhibitors and interactions commonly occur via metabolizing enzymes (i.e., cytochrome P450 isoenzyme superfamily)

or drug transporters (i.e., P-glycoprotein). However, the clinical relevance of these interactions may vary upon the azole involved and upon the target drug. Azoles may also be under the influence of and become targets of metabolic drug-drug interactions.

Pharmacokinetic interactions

The changes in absorption or elimination of the interacting drug and the antifungal interactions of drug absorption are observed (34). Ketoconazole and itraconazole are weak bases. They are ionized only at a low pH. Consequently, dissolution and absorption of these compounds is heavily dependent on acidic gastric conditions in the stomach (35–36). Drugs that increase gastric pH (e.g., H2 antagonists, proton pump inhibitors) slow the dissolution of the solid dosage forms and decrease the drug available for absorption in the intestinal lumen. Pharmacokinetic studies have documented 30–60% reductions in serum itraconazole concentrations in healthy volunteers administered itraconazole capsules with either famotidine or omeprazole (37–39). Absorption of the solution formulation of itraconazole, however, is not substantially reduced by drugs that increase gastric pH. Antacids, metal ion-containing drugs and vitamin supplements can also slow dissolution and absorption of ketoconazole or itraconazole through binding or chelating interactions that impair transport of the drug across the intestinal epithelium (40).

Two mechanisms have been identified as important modulators of presystemic clearance for azole antifungals such as ketonazole and itraconazole. The first mechanism is P-glycoprotein (P-gp), a versatile drug transporter. Ketoconazole or itraconazole can be both substrates and inhibitors of the P-gp, making the effects of P-gp efflux on azole absorption difficult to predict in individual patients (41). The second major mechanism of presystemic clearance of azole antifungal involves intestinal metabolism of lipophilic molecules by the cytochrome P450 (CYP) 3A4 enzymes, resulting in inactive and active metabolites. Similar to P-gP, azole antifungals are both substrates and inhibitors of CYP3A4 enzymes, suggesting that P-gP and CYP3A4 work in a coordinated fashion to prevent the absorption of xenobiotics. The degree of intestinal CYP3A4 differs from patient to patient and is not under coordinate expression of CYP3A4 in the liver, which is the principal site of drug metabolism and (presumably) clinically significant interactions affecting drug metabolism (42–43).

Cytochrome P450 and antifungal drug interactions

Phase I oxidative reactions are an important mechanism for the biotransformation of azole antifungals. Most oxidative reactions are catalyzed by a superfamily of mixed function mono-oxygenases called the cytochrome P40 system (44). Clinically significant genetic polymorphisms in CYP metabolism have been described for CYP2D6, CYP2C9, and CYP2C19. The frequency of polymorphisms also differs among different racial populations. Voriconazole pharmacokinetics, for example, is clearly affected by polymorphisms in CYP2C19 (45). Subpopulations of patients who are homozygous poor metabolizers through the CYP2C19 pathway may experience, on average, four-fold higher serum concentrations of voriconazole compared to other patients who are heterozygous extensive, or homozygous extensive metabolizers (majority of the population) (46).

Ketoconazole and itraconazole are highly lipophilic, their clearance is heavily dependent upon metabolism through several CYP 450 pathways including CYP 3A4. Fluconazole, on the other hand, is relatively less lipophilic and requires less CYP transformation at lower dosages (<200 mg/day) for clearance from the body. Co-administration of azoles with drugs that induce or accelerate strongly CYP-450 metabolism, particularly CYP 3A4, can result in low or undetectable levels of the azole antifungal (47–49). Higher antifungal dosages (particularly of ketoconazole, itraconazole, voriconazole) cannot overcome this interaction.

The most important drug interactions seen with azole antifungals typically arise from the inhibition of CYP 3A4, which plays a critical role in the metabolism of a broad array of drug therapies used for cardiovascular disease, endocrine disorders including hyperglycemia, anaesthesia, psychiatric disorders, epilepsy, cancer chemotherapy, and treatment of infectious diseases.

Metabolite profiles of major azole antifungals

Characterization of reactive and pharmacologically active metabolites is critical to design new drug candidates with improved metabolic stability, toxicological profile and efficacy. Metabolite identification in the preclinical species used for safety evaluation is required in order to determine whether human metabolites have been adequately tested during non-clinical safety assessment (50). Azole antifungals currently marketed are metabolized to some degree by the CYP P450 system. Ketoconazole is extensively metabolized into several inactive metabolites in the liver and the metabolites primarily excreted in bile (51), while fluconazole is mainly excreted in urine as the unmetabolized form (approximately 80%) (52). Fluconazole is less affected by CYP3A4 inducers (53), so fluconazole may be an alternative for patients receiving co-medicated CYP3A4 inducers. Voriconazole is extensively metabolized by CYP2C19, CYP2C9 and CYP3A4. Inducers or inhibitors of these isoen-

zymes may increase or decrease voriconazole plasma concentrations. Allelic polymorphisms of CYP2C19 have been shown to be the most important determinants of the clearance of voriconazole (54–56). Coadministration of a potent CYP3A4 inhibitor leads to a higher and prolonged exposure with voriconazole that might increase the risk of adverse drug reactions in the short term. Simultaneously with voriconazole, pharmacotherapy monitoring and dosage adjustment for these drugs should be implemented accordingly. The major metabolite of voriconazole, the voriconazole N-oxide, also inhibits the metabolic activity of CYP2C9, CYP3A4, and to a lesser extent, CYP2C19. Inhibitors or inducers of these three enzymes may increase or decrease voriconazole plasma concentrations, respectively. In addition, there is potential for voriconazole and its major metabolite to increase the plasma concentrations of other drugs metabolized by these enzymes (57).

Itraconazole is metabolized predominately by the cytochrome P450 3A4 isoenzyme system (CYP3A4), resulting in the formation of several metabolites, including hydroxyitraconazole, the major metabolite. It is metabolized *in vitro* to three inhibitory metabolites: hydroxy-itraconazole (OH-ITZ), keto-itraconazole (keto-ITZ), and N-desalkyl-itraconazole (ND-ITZ).When itraconazole in plasma is measured by a bioassay, values reported are approximately 3.3 times higher than those obtained by HPLC (high performance liquid chromatography) due to the presence of the bioactive metabolite, hydroxyitraconazole. Itraconazole and its major bioactive metabolite, hydroxyitraconazole, are inhibitors of CYP3A4 (58).

Posaconazole was discovered as a possible active metabolite of SCH 51048, an earlier lead. The analysis suggested that the active metabolite is a secondary alcohol with the hydroxyl group at the aliphatic side chain of SCH 51048 (59).

Prescribing azole antifungal drugs is an iterative process, in which physicians initially prescribe a standard dose of medication and then adjust the dose or choice of medication in response to the observed toxic, metabolic and therapeutic response (60–63). The above consequences suggest the importance of the determination of azole antifungals and their active metabolites in clinical practice for achieving optimal individualized treatment.

The pharmacodynamics and pharmacokinetics of azole drugs as indicated by the data above make the need for characterization of all their metabolites even more evident. Availability of protocols for qualitative and quantitative characterization of all their metabolites will have many applications for therapeutic drug monitoring, bioequivalence, toxicological and all related studies.

Isolation of metabolites

It is often necessary to isolate metabolites from biological matrices. This can be achieved in several ways, but most of them need a sample preparation step. Sample preparation has to be applied before the separation step, which is usually a chromatographic technique, to prevent the equipment from being clogged, contaminated or damaged. In the isolation part the purpose is to separate the analytes of interest from the matrix, and an enrichment of the analytes is often necessary, to achieve a good result later in the detection step. The problem is to avoid removing active metabolites. This is very difficult to achieve without knowing what kind of compound or compounds can be isolated. Ways to achieve this is to use complementary methods or a general method. Traditionally, liquid-liquid extraction (LLE) has been used for sample preparation. Liquid-liquid extraction is a mature and robust method, however there are some drawbacks such as: it is time consuming, large amounts of hazardous organic solvents are used and it is very difficult to automate. The biggest disadvantage with LLE is that it is not suitable for reversed phase liquid chromatography (RP-LC) without further reprocessing of the samples.

Solid phase extraction (SPE) is another sample preparation technique that has been growing in popularity during the last twenty years. Nowadays, SPE is the most frequently used sample preparation technique, mainly because SPE is easily automated and has a wide range of applications, has high reproducibility and consumes low amounts of organic solvents. Solid phase extraction can be divided into four groups: reversed-phase (RP), normal-phase (NP), ion exchange (IE) and adsorption SPE. In SPE the analyte is trapped in the sorbent and thereafter desorbed and eluted with a small portion of organic solvent. Solid phase extraction can be a non-specific method and a wide range of compounds can be isolated using one sorbent material. LLE would be used for isolation of a wide range of compounds with different hydrophobicity. However, with the introduction of short columns in liquid chromatography (LC) and the selectivity of the mass spectrometer (MS), throughput of samples is again more and more limited by the time required for sample pretreatment. Therefore, various systems have been developed in order to integrate sample pretreatment with the separation and detection technique. Basically, three possibilities have been proposed for integrated sample pretreatment in the analytical procedures, i.e., at-line, on-line and in-line. The at-line coupling involves sample preparation by a robotic device and an autoinjector to inject the extracts into the analytical instrument. No direct stream of liquid between extraction unit and analysing unit is present. Moreover, not the entire extract is transferred to the analysing instrument. In contrast to on-line procedures, application of in-line systems implies the direct injection of

the sample into the analytical instruments. Since the sample pretreatment is a time consuming procedure, various systems have been developed to couple sample preparation with the separation/detection/quantitation step in order to speed up the procedure and to eliminate error prone handling. Less common integrations offering high throughput potential such as SPE-GC (Solid-Phase Extraction-Gas Chromatography), SPME-LC (Solid-Phase Microextraction-Liquid Chromatography), SPE directly coupled to Mass Spectrometry (MS) and turbulent-flow chromatography (or extraction). Thus chromatographic and non-chromatographic techniques can be used for the isolation of metabolites prior to their characterization.

Characterization of metabolites

LC-DAD (Liquid Chromatography with Diode Array Detector) is useful for quantitation convenience with accurate results, but has limitations pertaining to qualitative (identification) data generation. It can be applied for quantitation of known metabolites which are sensitive to UV light. GC-MS (Gas Chromatography-Mass Spectrometry) is another technique useful for the identification and quantitation of volatile and semi-volatile organic compounds in complex biological mixtures. It determines molecular weights and sometimes also the elemental compositions of unknown organic compounds in complex mixtures. GC-MS has disadvantages like co-elution of analytes, higher degree of expertise required than most other instrumentation, sample carryover, multi-component compound interferences with individual analytes. It has limited applications in biomedical analysis. Determination of positional substitution on aromatic rings is often difficult by GC-MS. Isomeric compounds cannot be distinguished by mass spectrometry, but they can often be separated chromatographically. The IR (infrared spectroscopy) can provide information on aromatic positional isomers that is not available with GC-MS. The NMR (Nuclear Magnetic Resonance spectroscopy) can provide detailed information on the exact molecular conformation. Numerous assays formerly based on GC-MS have been transferred to LC-MS (Liquid Chromatography-Mass Spectrometry due to reduced sample preparation efforts, better sensitivities and new options for the mass spectrometric identification of compounds including non-volatile compounds. More versatile samples can be handled by LC-MS. It has limitations like availability and cost effectiveness, more tedious automated sample preparation than GC-MS and non-applicability for stereochemical identification. Amongst the mentioned techniques LC-MS is the most efficient, accurate and sensitive method for biomedical analysis. Another powerful method for structure elucidation as well as quantitation is to couple HPLC with MS and NMR. HPLC-NMR-MS in bioanalytical research could become a fast and powerful tool that provides lots of structural information, provided future developments improve the sensitivity of NMR detection. HPLC-NMR-MS can be used at an early stage for the identification of known compounds. Still, there are several operational conflicts to overcome when coupling HPLC with NMR and MS, such as solvent compatibility, instrumental sensitivity and magnetic field effects. However, the major obstacle for most research groups would probably be the price of this hyphenated instrumentation, location and the expertise needed for operations.

High performance liquid chromatography-mass spectrometry

Among the wide array of analytical techniques and methods, LC–MS is without question the preeminent technique for the analysis of expected analytes in complex mixtures without prior component isolation. It is also widely used for the determination of unknowns, due to the complementary structure information generated by multiple stage MS experiment (64–65). However, LC–MS on its own is insufficient to unequivocally identify some unknowns because LC–MS is unable to distinguish between co-eluting geometrical or optical stereoisomers (66–67). It is blind to compounds that can undergo severe thermal degradation or cannot be effectively ionized in the ionization interface. Using LC–MS in a synergistic way for rapid and definite identification of unknowns has shown great promise. It has had an increasingly significant impact on the bioanalysis development process over the past several decades. Advances in the design and efficiency of the interfaces, that directly connect separation techniques with mass spectrometers, have afforded new opportunities for monitoring, characterizing, and quantification of metabolites. The structures of the major metabolites were assigned for some optically active antifungal azoles as 4-hydroxy-2-imidazolidinone and/or 5-hydroxy-2-imidazolidinone, based on HPLC and LC/MS/MS analyses. These hydroxylated compounds were prepared by reduction of the corresponding imidazolidinediones, and confirmed to be identical to the metabolites by HPLC (68).

Nuclear magnetic resonance spectroscopy

In many cases NMR is needed for unambiguous structure determination, especially for the stereo-specific identification of unknown bioactive compounds that may be of interest for the development of pharmaceuticals and functional foods. NMR is the most powerful spectroscopic tool for obtaining structural details of complex metabolites. The structure assignment of all isomers is possible by considering chemical shifts, coupling constant, and integration ratios

of the NMR data. Conventionally, NMR spectroscopic analysis has required time consuming isolation and purification steps in order to acquire NMR spectra on an individual component. The effect of voriconazole on metabolic profiles of yeast species and metabolites was identified by two-dimensional correlation NMR spectra, and relative peak integrals were calculated from one-dimensional 1H NMR spectra (69).

High performance liquid chromatography – nuclear magnetic resonance spectroscopy

This coupling eliminates the need for extensive sample purification, and increases the NMR capability of solving structural problems of complex mixtures (70). During the past decade, LC–NMR has been successfully applied to the analysis of mixtures of drug related and biological origins, drug metabolites, plant metabolites, inter-converting labile natural products and in the identification of cis/trans isomers or other possible co-eluting stereoisomers (71–75). However, an inherent drawback of NMR is its low sensitivity, and its inability to detect some functional groups having poor or non-existent magnetic properties (e.g. SO_4, NO_2) (76). The lack of sensitivity makes the on-flow LC–NMR measurement of minor constituents impossible, and hinders the direct access of 13C signal that provides fundamental information for structure elucidation. Furthermore, reverse phase LC separations with multiple protonated solvents are employed, and solvent gradients are used. Thereby, the capability of the determination of an unknown structure by LC–NMR alone is limited. Even though LC–NMR has been investigated (77–78), commercially available instruments were not launched until the last decade. This long development time of LC–NMR may be attributed to the intrinsic low sensitivity of NMR spectroscopy. With recent technical advances in high resolution NMR and NMR probe development, online LC–NMR hyphenation becomes technically applicable (79).

High performance liquid chromatography – nuclear magnetic resonance spectroscopy – mass spectroscopy

MS rapid screening and preliminary structure investigation, followed by supplementary NMR structure determination, has become a typical structure elucidation protocol in clinical, biological and natural product research. However, data correlation based on independent LC–MS and LC–NMR results of the same sample is sometimes difficult, due to possibly different chromatograms obtained by the two systems. To avoid this ambiguity, MS and NMR are combined with one LC to operate as LC–NMR–MS, and this combination has attracted considerable investigation interest. LC–NMR–MS allows for the acquisition of MS and NMR data simultaneously in a single LC run, which provides inclusive analysis of a complex biological matrix through the real time comparison and complementation of NMR and MS data. Because MS analysis can provide the number of exchangeable protons of a compound by comparing the MS data in deuterated and non-deuterated solvents, it helps the determination of, for example, the number of hydroxyls in an unknown (80). On the other hand, the acidic proton exchange also complicates MS data interpretation. In the past decade, online coupled liquid chromatography–nuclear magnetic resonance spectroscopy–mass spectrometry (LC–NMR–MS) has emerged as a powerful tool for the detection and identification of known and, more importantly, emerging compounds in complex clinical, pharmaceutical samples and nature product extracts, due to the complementary information provided by the two detectors for unambiguous structure elucidation (81). Metabolite profiling of serum samples of posaconazole at selected time intervals pinpointed the peak that was suspected to be the active metabolite. The serum was harvested and the metabolite was isolated by extraction and semi-preparative HPLC. LC-MS/MS analysis suggested that the active metabolite of posaconazole is a secondary alcohol with the hydroxyl group in the aliphatic side chain of SCH 51048.

Gas chromatography-mass spectrometry

Highly sophisticated instrumentation, such as MS attached to a GC or HPLC, is an inevitable tools in the identification of minor components (drugs, impurities, degradation products, metabolites) in various matrices (82).

Extraction – nuclear magnetic resonance spectroscopy

It is an efficient and rapid method to obtain structural information on metabolites without prior separation. Crude extracts of *in vitro* microsomal incubations can be analysed by NMR spectroscopy. Depending on the complexity of parent compound, signal overlap and the relative abundance of the individual components, extraction-NMR has the potential to provide information for unambiguous structure elucidation of major metabolites. It is possible to re-use the extraction-NMR sample and proceed with traditional methods of analysis (83).

Conclusion

It is mandatory in the individualised therapy of antifungal azoles to know the analytical profiles for metabolites. Isolation, characterization, identification and quantitation of azole metabolites are required for

acquiring and evaluating data that generates biological safety. To isolate, identify and quantify the azole metabolites, a variety of instrumental analytical techniques described above may be characteristically used in biomedical research. Online LC–NMR–MS may be a powerful tool for solving identification and structure-related problems that a LC–MS or LC–NMR alone cannot handle. A lot of drug monitoring, bioequivalence, toxicological and all related studies about antifungal azole metabolites are needed. The azole metabolites data must be generated in times to come to provide safe and reliable treatment of the ever growing number of antifungals in service of mankind.

Acknowledgements: Authors wish to acknowledge the support received from the management and Principal of Bharati Vidyapeeth College of Pharmacy, Kolhapur for providing facilities and technical assistance.

References

1. Eicher T, Hauptmann S. The chemistry of heterocycles: structure, reactions, syntheses, and applications. Wiley, 2nd ed. VCH, 2003: ISBN 3527307206.
2. Lepesheva GI, Virus C, Waterman MR. Conservation in the CYP51 family: role of the B Helix/BC loop and helices F and G in enzymatic function. Bio 2003; 42: 9091–101.
3. Pfaller MA, Riley J, Koerner T. Effects of terconazole and other azole antifungal agents on the sterol and carbohydrate composition of candida albicans. Diag Micro Infect Dis 1990; 13: 31–5.
4. Majdic G, Parvinen M, Bellamine A, Harwood HJ, Jr Ku WW, Waterman MR, et al. Lanosterol 14 demethylase (CYP51), NADPH cytochrome P450 reductase and squalene synthase in spermatogenesis: late spermatids of the rat express proteins needed to synthesize follicular fluid meiosis activating sterol. J Endocrinol 2000; 166: 463–74.
5. Cotman M, Jezek D, Fon TK, Frangez R, Rozman DA. Functional cytochrome P450 lanosterol 14 demethylase CYP51 enzyme in the acrosome: transport through the golgi and synthesis of meiosis activating sterols. Endocrinol 2004; 145:1419–26.
6. Albengres E, Louet H, Tillement JP. Drug interactions of systemic antifungal agents. Drug Safety 1998; 18: 83–97.
7. Hoesley C, Dismukes WE. Overview of oral azole drugs as systemic antifungal therapy. Semin Resp Crit Care Med 1997; 18: 301–9.
8. Kauffman CA, Carver PL. Use of azoles for systemic antifungal therapy. Advan Pharmacol 1997; 39: 143–89.
9. Grant SM, Clissold SP. Fluconazole: a review of its pharmacodynamic and pharmacokinetic properties, and therapeutic potential in superficial and systemic mycoses. Drugs 1990; 39: 877–916.
10. Goa KL, Barradell LB. Fluconazole: an update of its pharmacodynamic and pharmacokinetic properties and therapeutic use in major superficial and systemic mycoses in immunocompromised patients. Drugs 1995; 50: 658–90.
11. Grant SM, Clissold SP. Itraconazole: a review of its pharmacodynamic and pharmacokinetic properties, and therapeutic use in superficial and systemic mycoses. Drugs 1989; 37: 310–44.
12. Boucher HW, Groll AH, Chiou C, Walsh TJ. Newer systemic antifungal agents. Drugs 2004; 64: 1997–2020.
13. Torres HA, Hachem RY, Chemaly RF, et al. Posaconazole: a broadspectrum triazole antifungal. Lancet Infect Dis 2005; 5: 775–85.
14. Donnelly JP, De Pauw BE. Voriconazole a new therapeutic agent with an extended spectrum of antifungal activity. Clin Microbiol Infect 2004; 10(1): 107–17.
15. Pfizer Australia Pty Ltd, product information, diflucan (fluconazole), 2006: version pfpdiflb 11206.
16. Schering Plough, Australia, product information: noxafil oral suspension (posaconazole), 2006: version 060310.
17. Pfizer Australia Pty Ltd, prescribing information: Vfend (voriconazole), 2006: version pfpvfend10406.
18. Sugar AM. Treatment of invasive aspergillosis, 2007: http://www.uptodate.com
19. Pfaller MA, Diekma DJ. Rare and emerging opportunistic fungal pathogens: concern for resistance beyond candida albicans and aspergillus fumigatus. J Clin Microbiol 2004; 42: 4419–31.
20. Poirier JM, Berlioz F, Isnard F, Cheymol G. Marked intra and inter patient variability of itraconazole steady state plasma concentrations. Therapie 1996; 51: 163–7.
21. Barone JA, Moskovitz BL, Guarnieri J, et al. Enhanced bioavailability of itraconazole in hydroxypropyl β cyclodextrin solution versus capsules in health volunteers. Antimicrob Agents Chemother 1998; 42: 1862–5.
22. Bodey GP. Azole antifungal agents. Clin Infect Dis 1992; 14 (1): S161–S169.
23. Espinel Ingroff A. Clinical relevance of antifungal resistance. Infect Dis Clin North Am 1997; 11: 929–44.
24. Documed AG. Arzneimittel Kompendium der Schweiz, 1992: http://www.kompendium.ch/.
25. Murray R. Role of antiaromatase agents in postmenopausal advanced breast cancer. Cancer Chemoth Pharmacol 2001; 48: 259–65.
26. Santen RJ, Harvey HA. Use of aromatase inhibitors in breast carcinoma. Endocr Relat Cancer 1999; 6: 75–92.
27. Santner SJ, Pauley RJ, Tait L, Kaseta J, Santen RJ. Aro-

matase activity and expression in breast cancer and benign breast tissue stromal cells. J Clin Endocrinol Metab 1997; 82: 200–8.

28. Bhatnagar AS, Brodie AM, Long BJ, Evans DB, Miller WR. Intracellular aromatase and its relevance to the pharmacological efficacy of aromatase inhibitors. J Steroid Biochem Mol Biol 2001; 76: 199–202.

29. Bisagni G, Cocconi G, Scaglione F, Fraschini F, Pfister C, Trunet PF. Letrozole, a new oral non-steroidal aromastase inhibitor in treating postmenopausal patients with advanced breast cancer. A pilot study. Ann Oncol 1996; 7: 99–102.

30. De Jong PC, Van de Ven J, Nortier HW, Maitimu Smeele I, Donker TH, Thijssen JH, et al. Inhibition of breast cancer tissue aromatase activity and estrogen concentrations by the third generation aromatase inhibitor voroconazole. Cancer Res 1997; 57: 2109–11.

31. Dixon JM, Renshaw L, Bellamy C, Stuart M, Hoctin Boes G, Miller WR. The effects of neoadjuvant anastrozole (arimidex) on tumor volume in postmenopausal women with breast cancer: a randomized, double blind, singlecenter study. Clin Cancer Res 2000; 6: 2229–35.

32. Takayama K, Zeitoun K, Gunby RT, Sasano H, Carr BR, Bulun SE. Treatment of severe postmenopausal endometriosis with an aromatase inhibitor. Fertil Steril 1998; 69: 709–13.

33. Wickman S, Sipila I, Ankarberg Lindgren C, Norjavaara E, Dunkel L. A specific aromatase inhibitor and potential increase in adult height in boys with delayed puberty: a randomised controlled trial. Lancet 2001; 357: 1743–8.

34. Roger JM, Jan WC, Nicole MA, Eliane MB, Jos GW, Paul E, et al. Pharmacokinetic drug interactions of azoles. Curren Fun Infec Rep 2008; 2: 20–7.

35. Gubbins PO, McConnell SA, Penzak SR, Piscitelli SC and Rodvold KA (editors). Antifungal agents drug interactions in infectious diseases. Humana Press: Totowa, NJ, 2001.

36. Lange D, Pavao JH, Wu J, Klausner M. Effect of a cola beverage on the bioavailability of itraconazole in the presence of H2 blockers, J Clin Pharmacol 1997; 37: 535–40.

37. Jaruratanasirikul S, Sriwiriyajan S. Effect of omeprazole on the pharmacokinetics of itraconazole. Eur J Clin Pharmacol 1998; 54: 159–61.

38. Kanda Y, Kami M, Matsuyama T, Mitani K, Chiba S, Yazaki Y, et al. Plasma concentration of itraconazole in patients receiving chemotherapy for hematological malignancies: The effect of famotidine on the absorption of itraconazole. Hematol Oncol 1998; 16: 33–7.

39. Lim SG, Sawyerr AM, Hudson M, Sercombe J, Pounder RE. Short report: The absorption of fluconazole and itraconazole under conditions of low intragastric acidity. Aliment Pharmacol Therapeut 1993; 7: 317–21.

40. Baciewicz AM, Baciewicz FA. Ketoconazole and fluconazole drug interactions. Arch Intern Med 1993; 153: 1970–6.

41. Hall SD, Thummel KE, Watkins PB, Lown KS, Benet LZ, Paine MF, et al. Molecular and physical mechanisms of first-pass extraction. Drug Metab Dispos 1999; 27: 161–6.

42. Lown KS, Kolars JC, Thummel KE, Barnett JL, Kunze KL, Wrighton SA, et al. Interpatient heterogeneity in expression of CYP3A4 and CYP3A5 in small bowel. Lack of prediction by the erythromycin breath test. Drug Metab Dispos 1994; 22: 947–55.

43. Kashuba DM, Bertino JS, Piscitelli SC and Rodvold KA (editors). Mechanisms of drug interactions. Drug Interactions in Infectious Diseases. Humana Press: Totowa, NJ, 2001.

44. Wrighton SA, Stevens JC. The human hepatic cytochromes P450 involved in drug metabolism. Crit Rev Toxicol 1992; 22: 1–21.

45. Ikeda Y, Umemura K, Kondo K, Sekiguchi K, Miyoshi S, Nakashima M. Pharmacokinetics of voriconazole and cytochrome P450 2C19 genetic status. Clin Pharmacol Ther 2004; 75: 587–8.

46. Johnston A. The pharmacokinetics of voriconazole. Br J Clin Pharmacol 2003; 56: 1: 1.

47. Nicolau DP, Crowe HM, Nightingale CH, Quintiliani R. Rifampin fluconazole interaction in critically ill patients. Ann Pharmacother 1995; 29: 994–6.

48. Bonay M, Jonville-Bera AP, Diot P, Lemarie E, Lavandier M, Autret E. Possible interaction between phenobarbital, carbamazepine and itraconazole. Drug Safety 1993; 9: 309–11.

49. Ducharme MP, Slaughter RL, Warbasse LH, Chandrasekar PH, Van de Velde V, Mannens G, et al. Itraconazole and hydroxyitraconazole serum concentrations are reduced more than tenfold by phenytoin. Clin Pharmacol Ther 1995; 58: 617–24.

50. Shuguang M, Chowdhury SK, Alton KB. Application of mass spectrometry for metabolite identification. Curr Drug Metab 2006; 7 (5): 503–23 16787159 (P, S, E, B).

51. Breckenridge A. Clinical significance of interactions with antifungal agents. Br J Dermatol 1992; 126: 39: 19–22.

52. Pfizer Inc. Prescribing information for DIFLUCAN®. Available from: URL:http://www.fda.gov/ohrms/dockets/ac/05/briefing/2005.

53. Niemi M, Backman JT, Fromm MF, Neuvonen PJ, Kivisto KT. Pharmacokinetic interactions with rifampicin: clinical relevance. Clin Pharmacokinet 2003; 42: 819–50.

54. Smith J, Safdar N, Knasinski V, Simmons W, Bhavnani SM, Ambrose PG, Andes D. Voriconazole therapeutic drug monitoring. Antimicrob Agents Chemother 2006; 50: 1570–2.

55. Pfizer Inc. Prescribing information for VFEND® (voriconazole) Tablets/ Injection.http://www.fda.gov/medwatch/SAFETY/2003/03MAR_PI/Vfend_PI.pdf.

56. Donnelly JP, De Pauw BE. Voriconazole – a new therapeutic agent with an extended spectrum of antifungal activity. Clin Microbiol Infect 2004; 10: 1: 107–17.

57. Isoherranen N, Kunze KL, Allen KE, Nelson WL & Thummel, KE. Role of itraconazole metabolites in

CYP3A inhibition. Drug Metab Dispos 32, 1121–31 (2004).

58. Janssen. Prescribing information for SPORANOX® (ITRACONAZOLE) INJECTION.http://www.fda.gov/medwatch/SAFETY/2004/jul_PI/SporanoxInj_PI.pdf.
59. Nomeir AA, Pramanik BN, Heimark, Bennett F, Veals J, Bartner P, et al. Posaconazole (Noxafil, SCH 56592), a new azole antifungal drug, was a discovery based on the isolation and mass spectral characterization of a circulating metabolite of an earlier lead (SCH 51048). J Mass Spectro 2008; 43 (4): 509–17.
60. Lillquist E, Sullivan CA. Legal regulation of the use of race in medical research. J Law Med Ethics 2006; 34: 535–51.
61. Harty L, Johnson K, Power A. Race and ethnicity in the era of emerging pharmacogenomics. J Clin Pharmacol 2006; 46: 405–7.
62. Emily M, Francois O. Conditional coalescent trees with two mutation rates and their application to genomic instability. Genetics 2006; 172: 1809–20.
63. Kim K, Johnson JA, Derendorf H. Differences in drug pharmacokinetics between East Asians and Caucasians and the role of genetic polymorphisms. J Clin Pharmacol 2004; 44: 1083–105.
64. Tomer KB. Separations combined with Mass Spectrometry. Chem Rev 2001; 101: 297–328.
65. Dachtler M, Glaser T, Kohler K, Albert K. Combined HPLC-MS and HPLC-NMR on-line coupling for the separation and determination of lutein and zeaxanthin stereoisomers in spinach and in retina. Anal Chem 2001; 73: 667–74.
66. Lindon JC, Nicholson JK, Sidelmann UG, Wilson ID. Directly coupled HPLC-NMR and its application to drug metabolism. Drug Metab Rev 1997; 29: 705–46.
67. Niessen WMA, Marcel Dekker Liquid Chromatography–Mass Spectrometry, second ed. New York, 1999.
68. Ichikawa, Yamada M, Yamaguchi M, Kitazaki T, Matsushita Y, Higashikawa K, et al. Optically Active Antifungal Azoles. XIII. Synthesis of stereoisomers and metabolites of 1-[(1R,2R)-2-(2,4-Difluorophenyl)-2-hydroxy-1-methyl-3-(1H-1,2,4-triazol-1yl)propyl]-3-[4-(1H-1-tetrazolyl)phenyl]-2-imidazolidinone. Chem Harm Bull 2001; 49: 9: 1110–19.
69. Muireann C, Jennifer B, Damla P, William AB, Himmelreich U, Kuchel PW, et al. Antifungal effects on metabolite profiles of medically important yeast species measured by nuclear magnetic resonance spectroscopy. Antimicrob Agents Chemother 2006; 50 (12): 4018–26.
70. Lommen A, Godejohann M, Venema DP, Hollman PCH, Spraul M. Application of directly coupled HPLC-NMR-MS to the identification and confirmation of quercetin glycosides and phloretin glycosides in apple peel. Anal Chem 2000; 72: 1793–7.
71. Albert K, Dachtler M, Glaser T, Händel H, Lacker T, Schlotterbeck G, et al. On-line coupling of separation techniques to NMR. J High Resol Chromatogr 1999; 22: 135–43.
72. Lindon LC, Nicholson JK, Wilson ID. Directly coupled HPLC–NMR and HPLC–NMR–MS in pharmaceutical research and development. J Chromatogr B 2000; 748: 233–58.
73. Wolfender JL, Ndjoko K, Hostettmann K. Liquid chromatography with ultraviolet absorbance-mass spectrometric detection and with nuclear magnetic resonance spectroscopy: a powerful combination for the on-line structural investigation of plant metabolites. J Chromatogr A 2003; 1000: 437–55.
74. Schaller F, Wolfender JL, Hostettmann K, Mavi S. New antifungal 'quinone methide' diterpenes from *bobgunnia madagascariensis* and study of their interconversion by LC/NMR. Helv Chim Acta 2001; 84: 222–9.
75. Albert K, Schlotterbeck G, Braumann U, Handel H, Spraul M, Krack G. Structure determination of vitamin A acetate isomers through coupled HPLC and 1H NMR spectroscopy. Angew Chem Ind Ed Engl 1995; 34: 1014–6.
76. Giinther H. NMR Spectroscopy, second ed. John Wiley & Sons. Chichester, UK, 1995.
77. Watanabe N, Niki E. Direct-coupling of FT-NMR to high performance liquid chromatography. Proc Jpn Acad Ser B 1978; 54: 194–9.
78. Holt RM, Newman MJ, Pullen FS, Richards DS, Swanson AG. High-performance liquid chromatography/NMR spectrometry/mass spectrometry: further advances in hyphenated technology. J Mass Spectrom 1997; 32: 64–70.
79. Lindon JC, Nicolson JK, Wilson ID. The development and application of coupled HPLC-NMR spectroscopy. Adv Chromatogr 1995; 36: 315–82.
80. Pullen FS, Swanson AG, Newman MJ, Richards DS. Online liquid chromatography/nuclear magnetic resonance mass spectroscopy – a powerful spectroscopic tool for analysis of mixtures of pharmaceutical interest. Rapid Commun Mass Spectrom 1995; 9: 1003–6.
81. Zheng Y. Online hyphenated liquid chromatography nuclear magnetic resonance spectroscopy–mass spectrometry for drug metabolite and nature product analysis. J Pharma Biomed Anal 2006; 40: 516–27.
82. Dimitrios T. Application of gas chromatography–mass spectrometry and gas chromatography–tandem mass spectrometry to assess *in vivo* synthesis of prostaglandins, thromboxane, leukotrienes, isoprostanes and related compounds in humans. J Chromatogr B 1998; 717: 201–45.
83. Gerhard U, Thomasm S, Mortishire SR. Accelerated metabolite identification by extraction-NMR. J Pharma Biomed Anal 2003; 32 (3): 531–8.

Received: August 18, 2008

Accepted: December 12, 2008