Stereoselective Determination of the Epimer Mixtures of Itraconazole in Human Blood Plasma using HPLC and Fluorescence Detection

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ABSTRACT Itraconazole is an antifungal drug widely used in a variety of fungal infections, which have become a significant public-health problem in recent decades. Itraconazole is a chiral drug consisting of two diastereoisomeric racemates, i.e., four stereoisomers. Data in the literature suggests that stereochemistry may play a significant role in the action and disposition of the drug and therefore stereoselective analytical methods for the determination of the drug in biological fluids are needed for the elucidation of that role. We report a stereoselective HPLC method that incorporates solvent extraction, the use of an internal standard, two chiral stationary phases in series, and fluorescence detection. The procedure is enantioselective and partially diastereoselective and provides the concentrations in blood plasma of the two epimer mixtures 2R,4S,2'R/2R,4S2'S and 2S,4R,2'R/2S,4R,2'S, respectively, each of which is a combination of the two epimers that differ in the configuration at the *sec*-butyl group. The analytical method has suitable sensitivity, recovery, precision, and accuracy. Analysis of the plasma of a human subject six hours after the oral administration of a single 200-mg dose of itraconazole showed a 3.4-fold difference between the concentrations of the epimer mixtures. The method has certain advantages over the published alternative procedure that uses LC-MS. *Chirality 23:495–503, 2011.*

KEY WORDS: Itraconazole; HPLC; bioanalysis; enantioselectivity; diastereoselectivity; fluorescence; antifungal; azole; stereoselectivity

INTRODUCTION

Fungal infections have become a major public health problem during the past ca. thirty years as a result of increased incidence of immunodeficiency due to the advent of HIV/AIDS, the widespread use of cancer chemotherapy, and immunosuppression in organ transplantation.[1,2] The "azoles" are important synthetic antifungal drugs and are so named for the presence of an imid*azole* or tri*azole* moiety in the molecule. Itraconazole (ITZ), a triazole, is an important and widely used agent in the treatment of a variety of fungal infections. Its chemical structure is chiral and includes three stereogenic centers and therefore eight stereoisomers are theoretically possible. However, the drug is defined and used clinically as a mixture of the two *cis* racemates, i.e., two pairs of enantiomers, four stereoisomers (Fig. 1a). Stereoisomers A and B in Fig. 1a are enantiomerically related, as are C and D. Diastereoisomers A and C are epimers since they have the same (2R,4S) configuration at the stereogenic centers of the dioxolane ring and differ in the configuration at only one (the *sec*-butyl center) of the three stereogenic centers in the molecules (it is worthwhile to recall the definition[3] of epimers: "Among molecules containing asymmetric atoms, epimers are diastereoisomers which differ in the configuration of any *one* (and *only* one) of the several asymmetric atoms").. Mixtures of A and C will henceforth be referred to as epimer mixture (2R,4S). Similarly, B and D have the same (2S,4R) configuration at the dioxolane ring and differ only in the configuration at the *sec*-butyl center, and will henceforth be referred to as epimer mixture (2S,4R). For the sake of completeness, it should be added that stereoisomers A and D are related as *diastereoisomers* but not as epimers, since they differ in the configuration at *two* of the three stereogenic centers. The same is true for the relationship of B and C.

There is considerable interest in the literature in the relationship between the pharmacokinetics and pharmacodynamics of ITZ, including the potential utility of therapeutic drug monitoring (TDM), i.e., the use of the concentrations of the drug in the blood of patients for guiding the physician in the optimization of therapy.[4,5,6,7,8] Despite such interest, however, little attention has been paid to the stereochemical aspects of the antifungal activity, toxicity, and disposition of ITZ. It has been reported in a U.S. patent[9] that epimer mixture (2S,4R) is 4-fold more potent than epimer mixture (2R,4S) against *Candida albicans* in vitro, but the antifungal activity of the four separate stereoisomers of ITZ has not been described. Kunze et al. studied the stereochemical aspects of the in vitro and in vivo metabolism of ITZ and found that only A and C are metabolized by Cytochrome P450 3A4 (CYP3A4).[10] The authors also reported that all four stereoisomers of ITZ induced a type II binding spectrum with CYP3A4 and all four inhibited the CYP3A4-catalyzed hydroxylation of midazolam. In addition, they also found that the human pharmacokinetics of ITZ was highly stereoselective, with epimer

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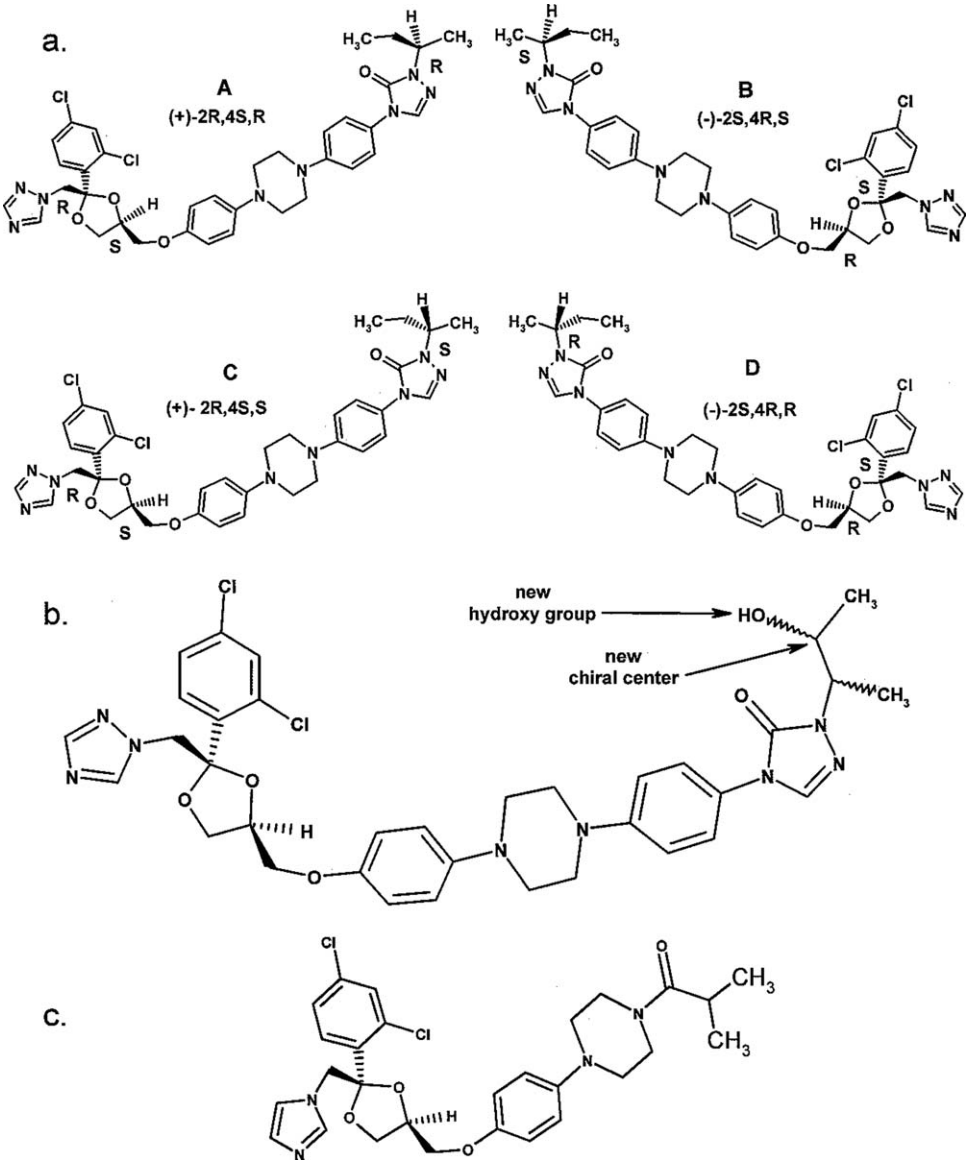
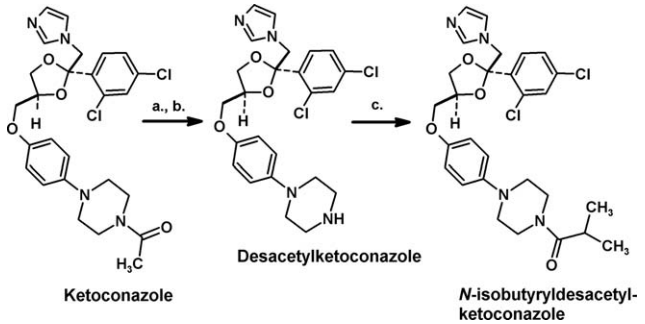


Fig. 1. The chemical structures of (a) the four stereoisomers of ITZ; A and B form one racemate and C and D form another; the third Cahn-Ingold-Prelog descriptor in the configurational specification of each stereoisomer is that of the stereogenic center in the sec-butyl group; (b) OH-ITZ. The hydroxyl group introduced during the biotransformation of ITZ to form OH-ITZ and the new stereogenic (chiral) center thus created are indicated; (c) IBDAK (the IS). The stereochemical wedge bonds in (b) and (c) imply only relative configuration.

mixture (2S,4R) reaching higher concentrations than epimer mixture (2R,4S).[10]

Limited work has been published on stereoselective analytical methodology needed for studies of the stereoselectivity of ITZ action and disposition. Breadmore and Thormann described a CE-based method for ITZ which produced two peaks, but the identity of the components giving rise to the peaks was not determined.[11] It is therefore not clear whether the conditions used[11] produced a separation of the racemates or of some other mixtures of stereoisomers. Another CE-based method[12] separated the four stereoisomers of ITZ but the technique was not applied to their determination in biological fluids. Using a custom-made CSP, one of the present authors and coworkers developed[13] a HPLC separation of the four ITZ stereoisomers, but the retention times were excessively long (total run time >100 min) and therefore the separation was unsuitable for pharmacokinetic studies involving a large number of samples. In the same study SFC was also examined and four peaks were obtained for ITZ, but two were only partially resolved, and the total run time was >60min.[13] Finally, in the above-cited study[10] on the stereoselective disposition of ITZ a LC-MS method was used. The chromatographic conditions employed produced 3 peaks for the drug and it was demonstrated that the epimers in the (2R,4S) mixture were not resolved. Moreover, the total run time was ca. 47 min and no internal standard was used in the quantification of the stereoisomers. The pharmacokinetics of the drug was characterized only for the two epimer mixtures, as mentioned above.[10]

The limited published data suggests that stereochemistry may play an important role in the pharmacodynamics and pharmacokinetics of ITZ and thus stereoselective analytical methods for the drug in biological fluids are of considerable interest. In the present article a HPLC method for the stereo-



Scheme 1. The synthesis of the internal standard. a. Ketoconazole, 85% powdered potassium hydroxide, 1-butanol, reflux 24hr. b. Remove solvent in vacuo, suspend residue in water, extract with chloroform, purify by HPLC. c. Isobutyric acid, HATU, DMF, N,N-diisoproylethylamine (for details see EXPERIMENTAL). The stereochemical bonds indicate only relative configuration; only the racemate was synthesized.

selective analysis of the two epimer mixtures in human blood plasma ("plasma" henceforth) using fluorescence detection is described.

EXPERIMENTAL

Chemicals

ITZ and hydroxyitraconazole were purchased from Janssen Pharmaceutical (Titusville, NJ, USA); ketoconazole and boric acid were from Sigma (St. Louis, MO, USA); HPLC-grade dioxane, isobutyric acid, *N,N*-diisopropylethylamine (99.5%), and anhydrous sodium sulfate were obtained from Aldrich (Milwaukee, WI, USA); trifluoroacetic acid (TFA, HPLC grade) was from Chem-Impex International Inc. (Wood Dale, IL, USA; O-(7-azabenzotriazol-1-yl)-N,N,N',N'-tetramethyluronium hexafluorophosphate (HATU) was obtained from Perseptive Biosystems (Warrington, England); sodium hydrogen carbonate (A.C.S. grade), anhydrous sodium sulfate (A.C.S. grade), HPLC-grade acetonitrile, ethyl acetate, hexane, methanol, and 2-propanol were purchased from Fisher Scientific (Pittsburgh, PA, USA); dimethylformamide (DMF, sequencing grade) was from Fisher; HPLC-grade methyl t-butyl ether (MTBE), ethanol, and denatured ethanol were purchased from Aldrich. Sodium hydroxide was obtained from JT Baker (Phillipsburg, NJ, USA).

The two epimer mixtures of ITZ were obtained via semipreparative HPLC separation of ITZ carried out by Chiral Technologies, Inc (West Chester, PA, USA). The semipreparative separation was based on our earlier analytical separation.[13] The separation used Chiralcel OD CSP (10mm x 50 cm) and ethanol as the mobile phase at 25° C; and detection was by UV at 270nm. The earlier-eluted peak was due to the dextrorotatory epimer mixture (2R,4S) and the later-eluting peak to the levorotatory epimer mixture (2S,4R) as determined with an optical-rotation detector[13]. The stereoisomeric purity of each mixture collected was >99%.

Synthesis of the Internal Standard (Scheme 1)

(±)-*N*-Isobutyryldesacetylketoconazole (IBDAK, Fig. 1c), used as internal standard (IS), was synthesized by acylation of *N*-desacetylketoconazole (DAK), in turn obtained by base-catalyzed deacetylation of ketoconazole according to a published procedure.[14] The acylation of DAK was carried out as follows: Isobutyric acid (38µl, 0.41 mmole), HATU, (155.9 mg, 0.41 mmole) were dissolved in 15 ml DMF in a 100-ml round-bottom flask with magnetic stirring. *N,N*-Diisoproylethylamine (143µl, 0.82 mmole) was added and the mixture was allowed to stand at room temperature for 3 min with magnetic stirring. (±)-DAK (200.7 mg, 0.41 mmole) was added and the solution was stirred overnight at room temperature. The solvent was removed under vacuum (ca. 3 mm Hg) at 30° and the residue was purified by gradient-elution HPLC. The stationary phase was ODS, contained in a 25 cm x 25 mm i.d. column. The gradient elution used a mobile phase consisting of two components: solvent A was 0.1% TFA in water and solvent B was 0.08% TFA in acetonitrile. The initial composition was 70% A and 30% B, final composition 50% A and 50% B. Total run time was 25 min, and detection was at 235nm. The eluted product was collected and concentrated at water aspirator pressure (ca. 36 mm Hg) at 35°C. The residue was dissolved in ethyl acetate and the solution was washed with 3x15 ml sodium hydrogen carbonate (3%) and then with brine (15 ml). The organic layer was dried over sodium sulfate, filtered, and evaporated at 35°C using a water aspirator. The residue was dissolved in dioxane (10 ml) and the solution was filtered and lyophilized. The yield of IBDAK was 160.2 mg (68.8%).

HPLC Instrumentation for ITZ analysis

The HPLC system consisted of a Shimadzu SIL-10AD auto injector, two Shimadzu LC-10AD pumps, and a Shimadzu RF-10AXL fluorescence detector. The chromatographic separation was achieved on a two-column system consisting of a Cyclobond I 2000 RSP (250 mm x 4.6 mm i.d.) (Astec, Whippany, NJ, USA) followed in series by a Daicel Chiralcel OD column of 4.6 mm id and 250 mm length (Chiral Technologies, Inc., Exton, PA). The columns were protected with a µBondapak Cyano guard column (Waters, Milford, MA). The fluorescence of the eluate was monitored at the excitation wavelength of 269 nm and emission at 374 nm.

Chromatographic conditions for ITZ analysis

The columns were maintained at 40° C using a model TCM column heater (Waters) and the separation was carried out using a gradient system. The mobile phase consisted of two components: solvent A was hexane and solvent B was a mixture of 2-propanol, denatured ethanol (i.e. ethanol containing 5% v/v 2-propanol), and acetonitrile, 42.5%, 42.5%, 15% v/v, respectively. In the gradient program the initial conditions were 75% A and 25% B. The linear gradient run was initiated at 0.01 min, and the final composition of 38% A and 62% B was reached over 22 min. At the end of the ramp the composition of the mobile phase was changed to 30% A and 70% B and this composition was maintained for 8 min. Initial conditions were then re-established. The flow rate was 1.5 ml/min throughout. These conditions provided two peaks for ITZ, the less retained material being the epimer mixture (2R,4S) while the longer-retained material was the epimer mixture (2S,4R), determined by injecting separately the individual epimer mixtures. The resolution R_S of the two peaks was determined according to the standard definition [10]. ITZ drug substance gave the two peaks in 1:1 ratio within experimental error (±3%).

Sample Preparation

A 500-µl plasma sample was placed into a 10-ml glass tube with a PTF-lined screw cap. A 100-µl aliquot of the internal standard solution (25 µg/ml in ethanol), 0.5 ml borate buffer (pH 10) and 5 ml of MTBE were added. The mixture was then mixed on a Barnstead-Thermolyne Labquake shaker (Fisher Scientific) for 20 min and centrifuged for five minutes at 10,000 rpm. The organic layer was transferred into a silanized tube and evaporated to dryness at 50° under a gentle stream of nitrogen. The residue was reconstituted in 150 µl of ethanol and 75 µl of the solution was injected in the HPLC.

Extraction Recovery

Extraction recovery from plasma was determined at 1.25 µg/ml for each stereoisomeric mixture, and the extraction procedure followed was as described above but with the IS solution replaced with an equal volume of the vehicle. Before evaporation of the extraction solvent the IS was added to the samples. Peak areas obtained upon HPLC analysis of the extracts were compared to the peak areas obtained upon analysis of unextracted samples containing equivalent concentrations. A similar procedure was used to determine the extraction recovery of the IS from plasma, adding ITZ to the extract before the evaporation.

Calibration Curves

Calibration curves were constructed using plasma samples spiked with ITZ at total ITZ concentrations of 50 ng/ml, 250 ng/ml, 1.00, 2.50, 5.00, 10.00 µg/ml (i.e. each stereoisomeric mixture having half the total concentration).

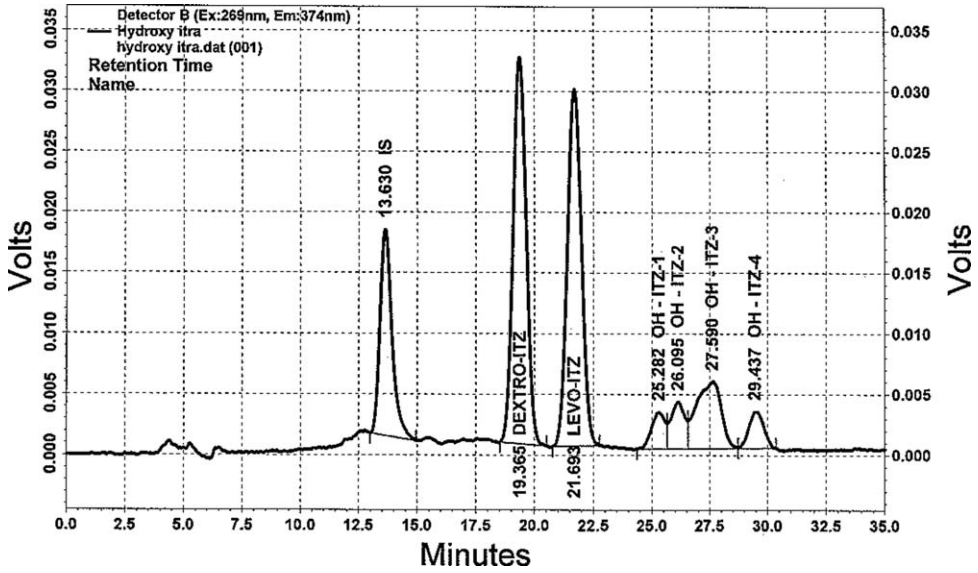


Fig. 2. HPLC chromatogram obtained upon analysis of a sample containing authentic standards of IS, ITZ, and OH-ITZ. The peaks are identified with IS for the internal standard, dextro and levo for epimer mixture (2R,4S) and epimer mixture (2S,4R), respectively, for ITZ, and with OH-ITZ for the hydroxy metabolite.

Intraday and Interday Precision and Accuracy

Intraday precision and accuracy were determined at total ITZ concentrations of 50 ng/ml, 100 ng/ml, 250 ng/ml, and 10.0 μg/ml and interday precision and accuracy at the concentrations of 50 ng/ml and 10.0 μg/ml.

Specificity

Drug-free plasma was analyzed without the addition of internal standard to determine potential interference from endogenous components. Five fluorescent drugs, ketoconazole, prazosin, propranolol, quinidine, and quinine, were examined for potential interference.

Limit of Detection and Limit of Quantification

The limit of detection (LOD) was determined by injecting decreasing amounts of ITZ into the HPLC system under the conditions of the analytical method. The lowest amount injected before reaching a signal-to-noise ratio of 3 was considered the LOD in the assay. The lower limit of quantification (LLOQ) was defined as the lowest plasma concentration of each epimer mixture that gave an intraday CV $\leq$ 15% and an intraday accuracy (deviation from target) of $\leq$ 10%.

Drug Administration

A healthy white 50-year-old male subject weighing 79.5 kg was administed a single oral dose of 200 mg ITZ (Sporanox® capsule). A plasma sample was drawn 6 hours after the dose and analyzed using the method described in the present article.

RESULTS

The Internal Standard

IBDAK, the internal standard, was synthesized using the reaction sequence shown in Scheme. 1. The yield was 69% and the matrix-assisted-laser-desorption/ionization-mass-spectrum (MALDI MS) of the product was consistent with the structure of IBDAK, giving an envelope of peaks corresponding to the cluster of protonated molecular ions (M+1) arising from the chlorine isotopes (35, 37) for the two chlorine atoms in the structure ($C_{28}H_{32}Cl_2N_4O_4$, M+1: 559, 561, 563). The purity of IBDAK thus obtained was found to be >98% by HPLC.

Chromatography

A stereoselective HPLC separation of ITZ was developed based on the Chiralcel OD chiral stationary phase (CSP). The separation produced two peaks, i.e. each peak was due to two stereoisomers contained in the drug (see **DISCUSSION**). The less-retained mixture was dextrorotatory while the longer-retained material was levorotatory.[13] Using vibrational circular dichroism it was shown that the configuration (at the dioxolane chiral centers) of the epimers of the first peak was 2R,4S (A and C, Fig.1a) and the configuration of the epimers of the second peak was 2S,4R (B and D, Fig.1a).[15] That is, in each epimeric mixture the two stereoisomers differ only in the configuration of the sec-butyl group (see **DISCUSSION**). However, the above separation proved inadequate, because the hydroxy metabolite of ITZ (OH-ITZ) (Fig. 1b) co-eluted with ITZ under these conditions. Chromatographic separation of the metabolite from the parent drug proved to be a challenging problem that was solved with the use of an additional column, Cyclobond I 2000 RSP containing a derivatized cyclodextrin CSP, in combination with gradient elution (Fig. 2). The peaks due to OH-ITZ elute well after those of ITZ and no longer interfere. The resolution R_S for the peaks of the two epimer mixtures was found to have the value of 2.05. The IS was also well separated from the ITZ and OH-ITZ peaks and eluted as a single peak, i.e., its enantiomers were not resolved.

Extraction Recovery

The mean recovery for epimer mixture (2R,4S) was 90.0±0.1% (n=5), for epimer mixture (2S,4R) 90.4±0.1% (n=5), and for the IS 90.5±0.0% (n=3).

Linearity

A linear relationship was found between the drug-to-internal standard peak-area ratios and the concentrations for both stereoisomeric mixtures. Calibration curves were linear for concentrations between 0.05 μg/ml and 10 μg/ml of total ITZ. The goodness of fit (r^2) for ITZ was consistently greater

TABLE 1. Intraday precision and accuracy (n = 6)

Concentration (per ml)	Precision %		Accuracy %	
	(+)-ITZ	(-)-ITZ	(+)-ITZ	(-)-ITZ
25ng	14.12	10.92	−4.00	−0.04
50ng	11.16	6.96	−5.60	−5.96
125ng	11.22	10.17	4.80	4.80
5.00μg	7.94	7.76	0.66	0.05

than 0.99. The mean for the slopes of five calibration curves obtained on five separate days was 1.080±0.076 for epimer mixture (2R,4S) and 1.073±0.073 for epimer mixture (2S,4R).

Precision and Accuracy

The precision and accuracy data obtained are presented in Tables 1 and 2.

LOD and LLOQ

The LOD was found to be 1.3 ng of each epimer mixture injected and the LLOQ was 25 ng/ml for each epimer mixture.

Typical chromatograms of plasma extracts are shown in Fig. 3.

Assay Specificity

No interference was seen when drug-free plasma samples were analyzed without the addition of the IS (Fig. 3a). Potential interference by the fluorescent compounds ketoconazole, propranolol, quinidine, and quinine was examined. Ketoconazole did not interfere but propranolol, quinidine, quinine, and prazosin were found to interfere in the procedure.

DISCUSSION

Numerous non-stereoselective HPLC methods for the determination of ITZ in plasma or serum have been published[16,17,18,19,20,21] but little has appeared on stereoselective analytical procedures. Thus, only one article[10] described an appropriate procedure for a stereoselective analysis but this method did not separate all four stereoisomers of ITZ and provided pharmacokinetic data only for the two epimer mixtures. In fact, no method has appeared for the stereoselective determination of all four stereoisomers in biological fluids, as discussed under INTRODUCTION above. A practical separation of the four stereoisomers with sufficient sensitivity and a reasonable run time for the processing of a large number of samples has indeed remained a challenging task, and this may be explained in part by the nature of the structure and stereochemistry of ITZ. The two chiral moieties (the dioxolane ring and the *sec*-butyl group, see Fig. 1a) are distant from each other in a large and elongated molecule, and the *sec*-butyl group is one of the smallest (non-hydrogen-isotope-based) chiral groups. These factors make the development of a practical chromatographic separation of the four stereoisomers challenging indeed. The published CE method[12] that separates the four stereoisomers of ITZ has not been applied to bioanalytical work and it is not clear whether it would have the required sensitivity and specificity.

In the present work a HPLC method was developed that is suitable for the determination of the epimeric mixtures of ITZ in plasma. In collaboration with a commercial supplier we obtained the two epimer mixtures using a semipreparative separation. The identity of the diastereoisomeric components in the separation was then established using vibrational circular dichroism as (+)-2R,4S,2'R, (+)-2R,4S,2'S, (-)-2S,4R,2'R, and (-)-2S,4R,2'S (where 2' refers to the *sec*-butyl stereogenic center, Fig. 1a).[15]

In an earlier study we found that the commercially available Chiralcel OD CSP gives two peaks for ITZ, with a total run time of ca. 25 min.[13] Using optical-rotation monitoring it was determined that the peak of shorter retention time was produced by the two dextrorotatory stereoisomers, i.e., epimer mixture (2R,4S) (A and C in Fig.1a) and the longer-retained peak was due to the two levorotatory diastereoisomers, i.e,, epimer mixture (2S,4R) (A and D, Fig. 1).[15] It is clear therefore that under the chromatographic conditions used the Chiralcel OD CSP does not separate the epimers differing in the configuration at the stereogenic center in the *sec*-butyl group (Fig. 1a). The separation obtained can be described as enantioselective since each set of enantiomerically related molecules is resolved (no enantiomerically related substances co-elute) but only partially diastereoselective, inasmuch as each set of epimers at the *sec*-butyl group stereogenic center remains unresolved. Obviously, some diastereoselectivity is achieved since stereoisomer A (Fig. 1a) is separated from D, and C is separated from B.

The Chiralcel OD-based chromatographic separation was used in the development of a stereoselective analytical method for the determination of the epimer mixtures of ITZ in plasma. An immediate problem was encountered in that OH-ITZ, the major metabolite of ITZ, coeluted with ITZ. This was clearly not acceptable since the circulating concentrations of the metabolite are significant[16] and would interfere with the determination of ITZ. The problem proved challenging inasmuch as the Chiralcel OD CSP did not separate ITZ from OH-ITZ under several conditions examined. The desired separation was finally achieved with the use of a derivatized beta-cyclodextrin CSP, Cyclobond I 2000 RSP, connected in front of and in series with the Chiralcel OD column. A gradient-elution-based separation was developed that produced the best separation of the metabolite from the parent drug without unduly extending the total run time (Fig. 2). The gradient also prevents the significant deterioration in the signal-to-noise ratio which would be caused by band broadening in isocratic elution from the 50-cm-long column set. Under these conditions good peak shapes were obtained; all components of interest (IS, ITZ, and OH-ITZ) are well separated from each other; and baseline resolution of the two epimer mixtures is achieved, with resolution R_S = 2.05 (Fig.2). We also found that the Cyclobond column alone did not separate the two ITZ epimer mixtures, that is, the ste-

TABLE 2. Interday precision and accuracy (n = 5)

Concentration (per ml)	Precision %		Accuracy %	
	(+)-ITZ	(-)-ITZ	(+)-ITZ	(-)-ITZ
25ng	11.45	8.24	2.12	−5.84
5.00μg	6.00	5.62	−0.44	0.10

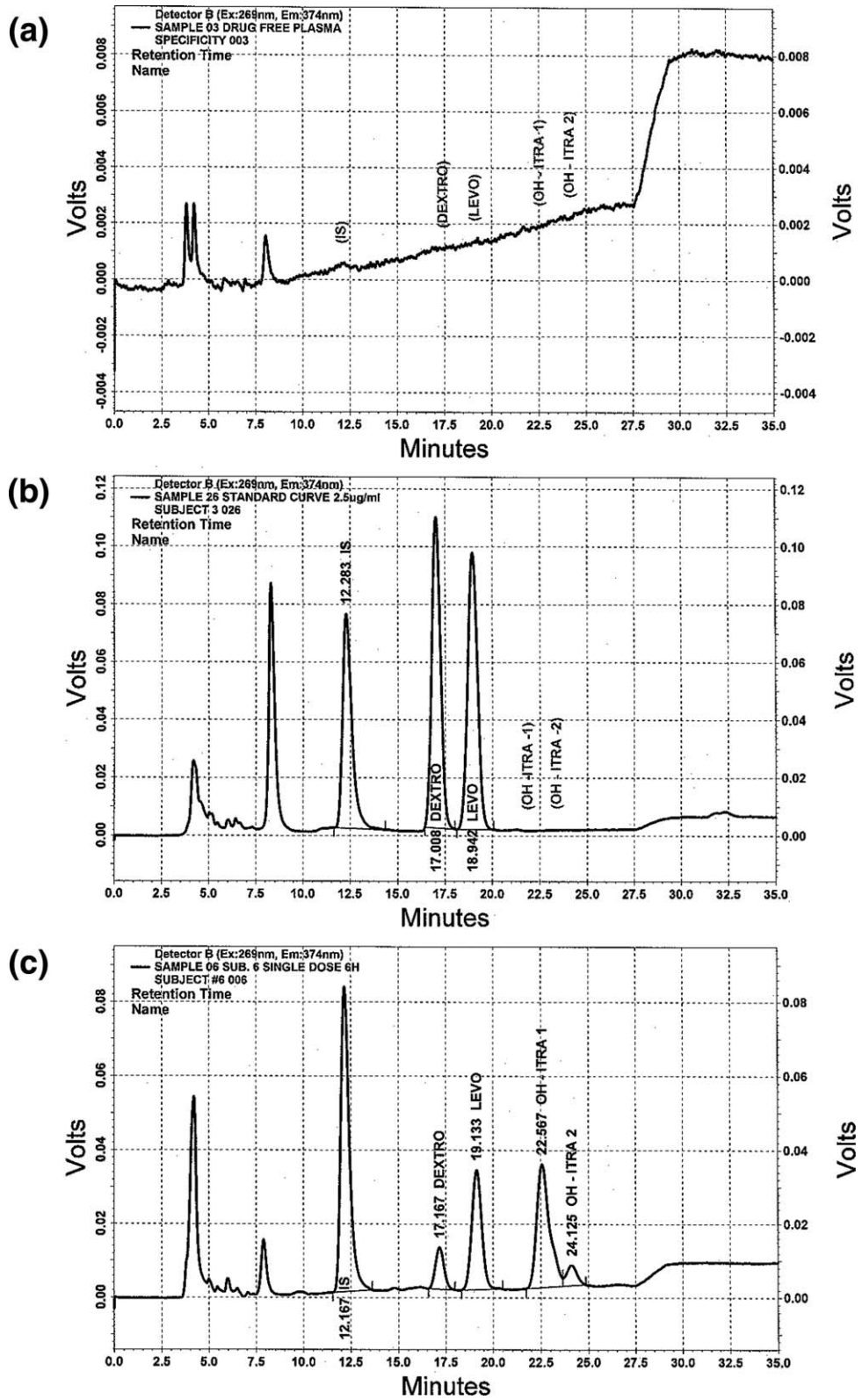


Fig. 3. HPLC chromatograms obtained upon analysis of (a) drug-free plasma without addition of IS; (b) plasma sample spiked with ITZ at 2.5 μg/ml; (c) plasma sample collected 6 hours after the administration of a 200-mg dose of ITZ to a male subject.

reoselectivity in our chromatographic system is provided by the Chiralcel OD CSP.

As seen in Fig. 2, authentic OH-ITZ gave an envelope of five incompletely resolved components. OH-ITZ contains four stereogenic centers but because the *trans* configuration at the dioxolane ring is excluded from the drug only eight stereoisomers are possible for the metabolite. Thus, the separation of the eight stereoisomers of OH-ITZ under the chro-

matographic condition used was incomplete. The chromatographic mechanism responsible for the separation of ITZ from OH-ITZ produced by the addition of the Cyclobond CSP is not clear. It is possible that selective inclusion complexation, a common mechanism for cyclodextrin-based retention and selectivity, produces the separation, but an alternative interaction of the hydroxyl group of OH-ITZ with the unmodified external hydroxyl groups on the cyclodextrin "cone" cannot be ruled out as the mechanism for the separation.

ITZ is a fluorescent compound and fluorescence has been frequently used in HPLC-based non-stereoselective determinations of the drug in biological fluids.[16,17,19,20,21] In the present procedure the fluorescence wavelengths were optimized for the conditions used but were similar to those used in other work.[19,20,21]

Identifying and obtaining a suitable internal standard was also a challenging task, for several reasons. First, fluorescence was the detection method used, which obviously requires a similarly fluorescent compound as IS; second, a difficult separation of two peaks of the drug and several peaks of the metabolite was involved which puts constraints on the retention properties of the IS; third, the commercially available racemic close analog of ITZ gave two peaks that were not separable from those of ITZ under a variety of conditions examined; fourth, no other suitable commercially available compound was found. We were therefore faced with the need to synthesize a suitable candidate for IS. Ca. 30 analogs of ITZ were synthesized, most of which were found to be entirely unsuitable as IS for one reason or another. However, one compound, IBDAK (Fig. 1c), a derivative of the related antifungal agent ketoconazole, did prove to be highly suitable as IS. This compound was synthesized (Scheme 1) via base-catalyzed *N*-deacetylation of ketoconazole and acylation of the resulting secondary amine with isobutyric acid, i.e, standard reactions of amides and secondary amines, respectively, and a good yield of the final product was obtained. Only the racemic mixture was synthesized but no resolution of its enantiomers was obtained under the chromatographic conditions used. Thus, the IS eluted as a single peak before the ITZ components (Fig. 2), with a suitable retention time. The chemical structure of the IS is similar to that of ITZ (Fig. 1), differing only in the nature of the moiety on N4 of the piperazine ring and in the identity of the 5-membered nitrogen-containing substituent on the dioxolane ring which is an imidazole group in the IS and a triazole in ITZ (Fig. 1). Overall, the extraction, chromatographic, and fluorescence properties of the IS are similar to those of ITZ and the compound is suitable for the role of IS, as demonstrated by the extraction-recovery, precision, and accuracy data obtained. IBDAK is not available from commercial sources.

The sample preparation used is simple and includes addition of the IS, adjustment of the pH, and a single liquid-liquid extraction, followed by evaporation of the solvent and reconstitution of the residue for injection into the HPLC system. Extraction recovery was 90% for all three components, i.e., the two epimer mixtures and the IS. Linear calibration curves were routinely obtained with $r^2 > 0.99$ and the curves for the epimer mixtures within each run were not significantly different. The standard deviations of the means of the slopes of the curves in five separate runs (different days) were <7.6%. The method demonstrated acceptable intra- and interday precision and accuracy (Tables 1 and 2).

No interference was seen when drug-free plasma samples were analyzed without the addition of the IS (Fig. 3a). Fig. 3b shows the chromatogram obtained upon analysis of a plasma sample spiked with ITZ. The procedure inherently incorporates considerable specificity due to several features. Extraction at pH 10 excludes acidic compounds ionized at that pH (e.g., the over-the-counter medications aspirin, ibuprofen, naproxen, etc.); the nonpolar extraction solvent excludes highly polar compounds; the chromatographic separation eliminates interference from compounds that do not coelute with the compounds of interest. In addition, fluorescence detection at specific excitation and emission wavelengths provides a great deal of specificity, since compounds that are non-fluorescent (e.g., acetaminophen) or that are fluorescent at other wavelengths remain undetected. Overall it is clear, therefore, that a large number of commonly used drugs do not interfere. In addition, we evaluated potential interference by several fluorescent compounds that were expected on the basis of their structures to follow ITZ during the sample preparation. Ketoconazole did not interfere but propranolol, quinidine, quinine, and prazosin were found to interfere in the procedure. Thus, the latter compounds should not be co-administered with ITZ when this analytical procedure is used for the determination of ITZ in biological fluids. Also, in all applications of the method potential interference from other agents that may be present should be evaluated.

Fig. 3c shows the chromatogram obtained after analysis of a plasma sample collected 6 hours after the administration of a single oral dose of 200 mg ITZ to a male subject. It is seen that the two epimer mixtures differ considerably in their plasma concentrations, in agreement with the data reported by Kunze et al.[10] Also noteworthy in Fig. 3c is the appearance of the peaks due to OH-ITZ. When these peaks are compared to the peaks produced by synthetic HO-ITZ (Fig. 2) it is seen that the metabolite formed in vivo consists of fewer stereoisomers than the synthetic material. Here too, our observations are in agreement with the earlier study.[10]

Based on literature reports of ITZ concentrations (sum of all four stereoisomers) in the serum or plasma of patients during multi-day dosing regimens or antifungal treatment, the LLOQ (25 ng/ml for each epimer mixture) of our analytical method provides suitable sensitivity for such studies. For example, treatment with a 200-mg daily oral ITZ dose during a 3-month period produced plasma steady state concentrations of total ITZ (sum of four stereoisomers) in the 800-1000 ng/ml range.[8] In another study,[22] 200mg intravenous ITZ (the approved formulation with hydroxypropyl-β-cylcodextrin) was administered in a 2-hour infusion to healthy subjects. The dose was given every 12 hr during days 1 and 2 and every 24 hr during the remaining 5 days. On day 7 the mean peak plasma concentration of total ITZ was 3349 ng/ml and the trough 916 ng/ml. In the only published report[10] on the pharmacokinetics of the two epimer mixtures of ITZ, after a 7-day administration of an oral solution of 100 mg ITZ to six healthy volunteers daily, the mean peak and trough concentrations of the (2R,4S) epimer mixture were determined as 356.3 ng/ml and 33.2 ng/ml, respectively. The corresponding values for the (2S,4R) epimer mixture were 520.8 ng/ml and 128.4 ng/ml, respectively. Based on non-stereoselective analytical methods used in clinical studies, it has been said that a trough value of at least 500 ng/ml (total) ITZ is required for the successful prevention or treatment of invasive fungal disease.[7,22] It is clear that our LLOQ readily meets this requirement and the stereochemical information

our method can provide may be important in defining the role of the various stereoisomers of ITZ in antifungal treatment and prophylaxis.

In single-dose pharmacokinetic studies the plasma concentrations achieved are of course significantly lower. For example, Templeton et al reported[23] that after an oral single dose of 100 mg ITZ administered to six healthy volunteers the mean peak plasma concentration of total ITZ was 255.4 ng/ml. After six hours the concentration declined to ca. 56 ng/ml and at 12 hr the concentration was ca. 35 ng/ml (estimated from the concentration vs. time curves). In the study[10]of the pharmacokinetics of the two epimer mixtures, in the only subject reported on individually the peak concentrations on day 1 of the 7-day study (100-mg oral ITZ solution) were 213.1 ng/ml and 69.9 ng/ml for the the (2S,4R) and the (2R,4S) mixtures, respectively, and the mean values for six subjects were similar to those of the single individual. However, the trough values after the first dose were below the LLOQ of our procedure and in fact the use of LC-MS was required in that study for their determination. The concentrations in the last two studies (both of which used the lower dose of 100 mg) should be contrasted to the results our study, in which 6 hr after the administration of a single 200-mg oral dose of ITZ the concentrations were 449 ng/ml and 134 ng/ml for the (2S,4R) and (2R,4S) epimer mixtures, respectively. It has been reported that ITZ displays dose-dependent pharmacokinetics and marked intra- and interpatient variability.[7] One or more of these factors, in combination with the higher, 200-mg, dose used in our study, may be the explanation for the considerably higher plasma concentrations achieved in our study compared to the concentrations found by Kunze et al.[10] who used 100-mg doses (it should be noted that the 200-mg dose is commonly used in pharmacokinetic studies and in antifungal therapy, and even 300-mg doses have been used[22] in some studies; moreover, in antifungal treatment daily oral doses of up to 400 mg can be used[24]). Concerning the limitations of our LLOQ in single-low-dose pharmacokinetic studies, it would seem that applying LC-MS detection to our method could provide the sensitivity needed for such studies. Such a combination of our use of a highly suitable internal standard with LC-MS detection would likely further enhance the utility and robustness of our analytical method.

An eventual full characterization of the stereoselectivity in the disposition of ITZ will require a suitable analytical method that resolves all four stereoisomers. Nevertheless, the procedure described in the present communication should prove a useful tool for studying the stereoselectivity of the disposition of the drug insofar as the chirality of the dioxolane ring is concerned. That the stereochemistry of the dioxolane ring of ITZ is important for its actions is demonstrated by the observation[10] that only the (2R,4S) stereoisomers (regardless of the configuration at the *sec*-butyl group) are metabolized by CYP3A4. Our method has some advantages over the other procedure[10] in the literature, e.g., a significantly shorter run time, the use of the considerably simpler and less costly fluorescence detector instead of LC-MS, and, importantly, the inclusion of an internal standard. However, LC-MS detection can provide higher sensitivity, which may be required in some studies using single-low-dose drug administration, as discussed above.

Our procedure may also be applicable to the analysis of drugs that have the same (or a very similar) dioxolane-based chiral structural element, e.g., in several other azole antifungal agents such as ketoconazole, terconazole, saperconazole, etc. Indeed, we recently applied a similar analytical procedure to the enantiospecific determination in plasma of ketoconazole, a drug administered as the racemic mixture, and the procedure appeared to provide the basis of a useful method for the determination of this drug (unpublished data).

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REFERENCES

1. Groll AH, Shah PM, Mentzel C, Schneider M, Just-Nuebling G, Huebner K. Trends in the postmortem epidemiology of invasive fungal infections at a university hospital. J Infect 1996;33;23–32.
2. Singh N. Trends in the epidemiology of opportunistic fungal infections: Predisposing factors and the impact of antimicrobial use practices. Clin Infect Dis 2001;33:1692–1696.
3. Mislow K. Introduction to stereochemistry. New York: W. A. Benjamin; 1965. p. 91.
4. Baietto L, D'Avolio A, Ventimiglia G, De Rosa FG, Siccardi M, Simiele M, Sciandra M, Di Perri G. Development, validation, and routine application of a high-performance liquid chromatography method coupled with a single mass detector for quantification of itraconazole, voriconazole, and posaconazole in human plasma. Antimicrob Agents Chemother 2010;54:3408–3413.
5. Timmers GJ, Kessels LW, Wilhelm AJ, Veldkamp AI, Bosch TM, Beijnen Jh, Huijgens PC. Effects of Cyclosporine A on single-dose pharmacokinetics of intravenous itraconazole in patients with hematologic malignancies. Ther Drug Monit 2008;30:301–305.
6. Yao M, Srinivas N. Bioanalytical methods for the determination of itraconazole and hydroxyitraconazole: overview from clinical pharmacology, pharmacokinetic, and metabolism perspectives. Biomed Chromatogr 2009;23:677–691.
7. Poirier J-M, Cheymol G. Optimization of itraconazole therapy using target drug concentrations. Clin Pharmacokinet 1998;35:461–473.
8. Havu V, Brandt H, Heikkilä H, Hollmen A, Oksman R, Rantanen T, Saari S, Stubb S, Turjanmaa K, Piepponen T. Continuous and intermittent itraconazole dosing schedules for the treatment of onychomycosis: a pharmacokinetic comparison. Brit J Dermatol 1999;140:96–101.
9. Koch P, Rossi RF Jr, Senanayake CH, and Wald SA, inventors, Sepracor, Inc., assignee. 2R,4S-Hydroxyitraconazole isomers. U.S. Patent 6,147,077, 2000 Nov 14.
10. Kunze ,KL, Nelson WL, Kharasch ED, Thummel KE, and Isoherranen N. Stereochemical aspects of itraconazole metabolism in vitro and in vivo. Drug Metab Disp 2006;34:583–590.
11. Breadmore MC, Thormann W. Capillary electrophoresis evidence for the stereoselective metabolism of itraconazole in man. Electrophoresis 2003;24:2588–2597.
12. Castro-Puyana M, Crego AL, and Marina ML. Separation and quantification of the four stereoisomers of itraconazole in pharmaceutical formulations by electrokinetic chromatography. Electrophoresis 2006;27:887–895.
13. Thienpont A, Gal J, Aeschlimann C, Felix G. Studies on stereoselective separations of the "azole" antifungal drugs ketoconazole and itraconazole using HPLC and SFC on silica-based polysaccharides. Analusis 1999;27:713–718.
14. Chapman DR, Bauer L, Waller DP, and Zanewald LJD. Synthesis of diastereomeric ketoconazole analogs .1. J Hererocyc Chem 1990;27:2063–2068.
15. Dunmire D, Freedman TB, Nafie LA, Aeschlimann C, Gerber JG, andGal. Determination of the absolute configuration and solution conformation of the antifungal agents ketoconazole, itraconazole, and miconazole with vibrational circular dichroism. Chirality 2005;17:S101–S108.
16. M. Poirier JM, Lebot M, Descamps P, Levy M, and Cheymol G. Determination of itraconazole and its active metabolite in plasma by column liquid-chromatography. Ther Drug Monit 1994;16:596–601.
17. Poirier JM and Cheymol G. A rapid and specific liquid chromatographic assay for the determination of itraconazole and hydroxyitraconazole in plasma. Ther Drug Monitor 1997;19;247–248.
18. Koks CH, Sparidans RW, Lucassen G, Crommentuyn KM, and Beijnen JH. Selective high-performance liquid chromatographic assay for itraconazole

and hydroxyitraconazole in plasma from human immunodeficiency virus-infected patients. J Chromatogr B 2002;767:103– 110.

19. Compas D, Touw DJ, and deGoede PN. Rapid method for the analysis of itraconazole and hydroxyitraconazole in serum by high-performance liquid chromatography. J. Chromatogr B Biomed Appl 1996;687:453–456.
20. Yao M, Chen L, and Srinivas NR. Quantitation of itraconazole in rat heparinized plasma by liquid chromatography-mass spectrometry. J. Chromatogr B Biomed Appl 2001;752:9–16.
21. Srivatsan V, Dasgupta AK, Kale P, Datla RR, Soni D, Patel M, Patel R, and Mavadhiya C. Simultaneous determination of itraconazole and hydroxyitraconazole in human plasma by high-performance liquid chromatography. J. Chromatog. A 2004;1031;307–313.
22. Mouton JW, van Peer A, de Beule K, Van Vliet A, Donnelly JP, Soons PA. Pharmacokinetics of itraconazole and hydroxyitraconazole in healthy subjects after single and multiple doses of a novel formulation. Antimicrob Agent Chemother 2006;50:4096–4102.
23. Templeton IE, Thummel KE, Kharasch ED, Kunze KL, Hoffer C, Nelson WL, Isoherranen N. Clin Pharmacol Ther 2008;83:77–85.
24. Bennett JE. Antimicrobial agents. In: Hardman JG, Limbird LE editors: Goodman and Gilman's the pharmacological basis of therapeutics. 10th Edition. New York: McGraw-Hill; 2001. p. 1304.