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Determination of ketoconazole in human plasma by high-performance liquid chromatography–tandem mass spectrometry

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Abstract

A simple, rapid and specific high-performance liquid chromatography coupled with tandem mass spectrometry (LC–MS–MS) has been developed and validated for the determination of ketoconazole in human plasma. The method used diethyl ether to extract the ketoconazole and the internal standard (I.S.) R51012 from alkalinized plasma sample. The LC separation was on a C_{18} column (50×3 mm, 5 μm) using acetonitrile–water–formic acid (75:25:1, v/v/v) mobile phase. The retention times were approximately 1.8 min for both ketoconazole and the I.S. The MS–MS detection was by monitoring 531.2→82.1 (*m*/*z*) for ketoconazole, and 733.5→460.2 (*m*/*z*) for the I.S. The dynamic range was from 20.0 to 10 000 ng/ml based on 0.1 ml plasma, with linear correlation coefficient of ≥0.9985. The run time was 2.5 min/injection. The recoveries of ketoconazole and the I.S. were 102 and 106%, respectively. The precision and accuracy of the control samples were with the relative standard deviations (RSDs) of ≤4.4% (n=6) and the relative errors (REs) from −0.6 to 1.4% for intra-day assay, and ≤8.6% RSD (n=18) and −1.4 to 0.9% RE for inter-day assay. The partial volume tests demonstrated good dilution integrity. Three freeze–thaw cycles, keeping plasma samples at ambient for 24 h, storing extracted samples at ambient for 24 h, and storing frozen plasma samples at approximately −20 °C for up to 2 months did not show substantial effects.

Keywords: Ketoconazole

1. Introduction

Ketoconazole (*cis*-1-acetyl-4-{4-[[2-(2,4-dichlorophenyl)-2-(1H-imidazol-1-ylmethyl)-1,3-dioxolan-4-yl]-methoxy]phenyl}piperazine, given in Fig. 1a), a synthetic imidazole antifungal, is effective for superficial fungal infections, genital candidosis and chronic mucocutaneous candidosis, and has been widely used in immunocompromised patients and advanced prostatic carcinoma [1–4]. When a single dose of 200 mg ketoconazole was orally administered by an adult, the mean peak concentration in plasma was typically 3–4.5 μg/ml [2]. A detailed review article on the clinical pharmacokinetics (PK) of this drug was published by Daneshment and Warnock in 1988 [3]. Lelawongs et al. argued that food would diminished the rate of ketoconazole absorption and the reduction of gastric acidity provided an enhancement of ketoconazole absorption [5]. However, Daneshment suggested that the diseases and drugs but not food decreased the bioavailability of ketoconazole [6]. For the measurement of

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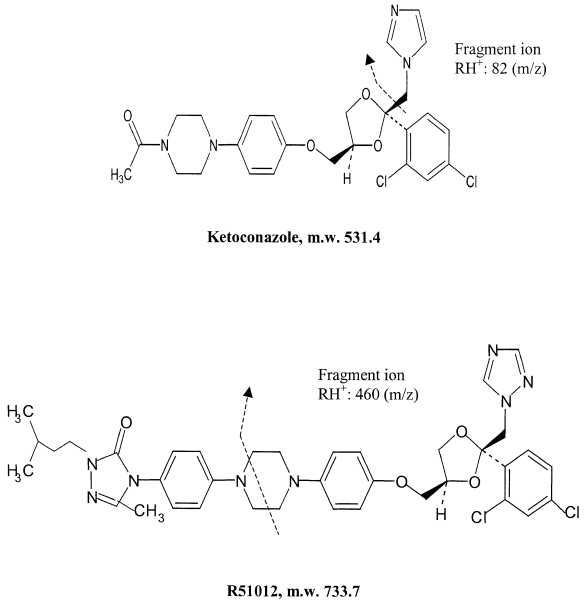

Fig. 1. Chemical structures of ketoconazole and R51012.

ketoconazole in biological fluids, a few microbiological methods have been used for different studies [7–13]. These bioassays established based on the antifungal activity of ketoconazole usually were non-specific and the results might be disturbed by other bioactive components or metabolites. Also the bioassays in the literature had the limited sensitivity typically with a lower quantitation limit of approximately 100 ng/ml. Since 1980, a number of high-performance liquid chromatography (HPLC) methods have been developed [14–22]. Alton reported the first HPLC method for the determination of ketoconazole in human plasma in which a reversed-phase column and UV detection were used to reach the lower quantitation limit of 100 ng/ml using 2 ml plasma [14]. A similar assay was described by Mannisto et al. [15]. These methods involved a multiple-step liquid–liquid extraction (extraction/back-extraction/extraction) procedure [14,15]. Andrews et al. presented an assay using C_{18} cartridge filtration for deprotenization and requiring a run time of 20 min [16]. None of the above HPLC methods [14–16] used an internal standard (I.S.) for the quantitation thus the extraction efficiency and recovery were crucial. Swezey et al. described a simple liquid–liquid extraction coupled with HPLC–fluorescence detection for the measurement of ketoconazole ranging from 0.10 to 2.5 μg/ml using 1 ml plasma in which an analogue of ketoconazole, R41300 (*cis*-ethyl-4-[4-{[2-(2,4-dichlorophenyl)-2-(1H-imidazol-1-ylmethyl)-1,3-dioxolan-4-yl]methoxy}phenyl]-1-piperazine carboxylate), was employed as the I.S. [17]. In 1983, Pascucci et al. presented a method which used a C_{18} solid-phase extraction cartridge to extract the analyte and the I.S., phenothiazine, and the method required a 0.5-ml plasma sample and achieved the lower quantitation limits of 0.20 μg/ml for UV and 0.04 μg/ml for fluorescence detection [18]. Badcock used a simple protein denaturation by acetonitrile for sample preparation and developed a HPLC–UV assay suitable for a quantification range of 0.1–20 μmol/l (approximately 50–1000 ng/ml), which needed only 20 μl plasma or serum but required a 15-min run time [19]. Later Turner et al. published a reversed-phase HPLC–UV method using terconazole as I.S. for determining ketoconazole in human serum with a lower quantitation limit of 50 ng/ml [20]. Riley and James employed clotrimazole as I.S. to track ketoconazole in the solid-phase extraction and chromatography and developed HPLC–UV methods for the determination of ketoconazole in the lung, liver, plasma and adrenal gland for the rat studies in which the detection limits (i.e., signal-to-noise ratio 3) were 200 ng/ml in plasma, 400 ng/g in lung or liver, and 25 μg/g in adrenal [21]. In 1988, Hoftman et al. described a method which used HPLC with electrochmical detection to improve the sensitivity by 20-fold against UV detection at 231 nm [22].

In previously published approaches, bioassays lacked specificity and HPLC assays typically required 10–20 min on-column separation, and both had either a relatively higher quantitation limit (50 ng/ml or higher) or a large sample size requirement. Supporting PK research often needs more sensitive, accurate, and specific methods. Potentially large numbers of samples in clinical trials need a rapid and reliable assay. An ideal method should have simple sample preparation, fast on-column separation, and sensitive and specific detection. Liquid chromatography coupled with mass spectrometry (LC–MS) has become such a tool which meets most of the above needs [23]. In many cases, highly specific mass spectrometric detection, especially using tandem mass spectrometry (MS–MS) just requires minimum

separation on column. This will greatly shorten the assay time and make it possible to analyze large quantities of samples within a tight time frame. To date no LC–MS–MS method has been reported for the quantification of ketoconazole in biological fluids.

In this paper we present a simple, rapid, and specific method with sufficient sensitivity for the determination of ketoconazole in human plasma. This method used R51012 as I.S. The structure of R51012, *cis*-4-(4-[4-[4-{[2-(2,4-dichlorophenyl)-2-(1H-1,2,4-triazol-1-ylmethyl)-1,3-dioxolan-4-yl]-methoxy]phenyl}-1-piperazinyl]phenyl)-2,4-dihydro-5-methyl-2-(3-methylbutyl)-3H-1,2,4-triazol-3-one, is shown in Fig. 1b. The assay used a simple liquid–liquid extraction and LC–MS–MS for separation and detection. With this procedure the recoveries for ketoconazole and R51012 were found to be 102 and 106%, respectively. The analysis time on LC–MS–MS was only 2.5 min. This method was validated for the determination of 20.0 to 10 000 ng/ml ketoconazole in human plasma using 0.1 ml sample and has been applied to support PK studies.

2. Experimental

2.1. Chemicals, materials and apparatus

Ketoconazole with purity 100% was purchased from USP (Rockville, MD, USA). R51012, with a purity of 99.51%, was from Janssen Biotech (Flanders, NJ, USA). The reference standards were used without further purification. Diethyl ether, high-purity solvent, Burdick & Jackson brand, was from Honeywell International (Muskegon, MI, USA). Formic acid, ACS reagent, was from Aldrich (Milwaukee, WI, USA). Ammonium hydroxide (29.7%), ACS plus, was from Fisher Chemicals (Fair Lawn, NJ, USA). Acetonitrile (ACN), methanol, and water were of HPLC grade and were from Fisher Scientific (St. Louis, MO, USA). Trifluoroacetic acid (TFA) was from Sigma (St. Louis, MO, USA). Blank human plasma with K_3-EDTA anticoagulant was from Biochemed Pharmacologicals (Winchester, VA, USA).

A dry ice–acetone bath was used for freezing aqueous portion during extraction. A Turbo Vap water-bath was used for evaporating organic solvent of extracts. The HPLC system including solvent delivery LC10ADVP, autoinjector SIL10ADVP, controller SCL10ADVP and column oven CTO10ASVP was from Shimadzu (Kyoto, Japan). The analytical column used was a BDS Hypersil C_{18} 50×3.0 mm (5 μm) from Keystone Scientific (Bellefonte, PA, USA). A Phenomenex C_{18} 4×3.0 mm was used as a guard column. A Supelco 0.5-mm in-line prefilter was also used to prevent particles from entering analytical column. The ionspray ionization-triple quadrupole mass spectrometer API 3000 from Sciex (Concord, Canada) was used for detection. Sciex sample control software version 1.4 and MacQuan version 1.6 with a power Macintosh computer were used for data acquisition and data analysis, respectively.

2.2. LC–MS–MS conditions

A BDS Hypersil C_{18} column (50×3 mm, 5 μm particles), connected with a guard column and prefilter, was used for sample analysis. The isocratic mobile phase used was acetonitrile–water–formic acid (75:25:1, v/v). The flow-rate of the mobile phase was 0.500 ml/min. The column was used at room temperature. The injection volume was 10 μl. An ACN–water–TFA (50:50:0.05, v/v) mixture was used as the injector wash solution. The autoinjector carryover was tested by injecting the upper limit of quantitation (ULOQ) extracted sample followed by an extracted blank or reagent blank. With this injector wash solution and rinse mode setting at 3, no carryover was observed.

The positive ionspray ionization mode was used to generate the molecular ions for the triple quadrupole mass spectrometric detection. The MS–MS monitoring pattern and conditions were optimized by infusing a ca. 0.2-μg/ml pure standard in methanol–water (1:1) containing 1% formic acid. The multiple reaction mode (MRM) was used to acquire the total ion counts at different time points. A high voltage of 4.5 kV was applied to the sprayer. The turbo gas temperature was 400 °C. The auxiliary gas flow was 8 l/min. The settings of nebulizer gas, curtain gas, and collision gas flows were 12, 10, and 8, respectively. All the gas used in this experiment was high purity nitrogen. The other optimized parameters

included the orifice voltage 46 V, the ring voltage 310 V, Q0 (−10 V), and RO2 (−58 V) for ketoconazole, and the orifice voltage 31 V, the ring voltage 290 V, Q0 (−10 V), and RO2 (−84 V) for R51012, respectively. The mass spectrum of ketoconazole is shown in Fig. 2. The protonated ketoconazole ion was observed at m/z of 531.2. Two major product ions with respective m/z values of 489.2 and 82.1 were formed by fragmentation of the precursor ion through the pathways of losing part I and further losing part II, as indicated in Fig. 2. For the I.S. R51012, the most abundant product ion was 460.2 from its singly charged precursor ion 733.5 (the mass spectrum not shown but the fragmentation pathway was briefly indicated in Fig. 1b). The detection was by monitoring the most intensive precursor→fragment transitions at m/z 531.2→82.1 for ketoconazole, and at m/z 733.5→460.2 for the I.S. R51012. The dwell time was 200 ms for both the analyte and the I.S. In this assay, both Q1 and Q3 quadrupoles were set at unit resolution. For each injection, the total data acquisition time was 2.5 min.

Peak areas of chromatograms were integrated and the ratios of the analyte/I.S. were calculated. The linear regression with a weighted $1/\text{concentration}^2$ was used to obtain calibration curve from standards and the regression equation of the calibration curve was then used to calculate the concentrations of quality control (QC) samples or practical clinical samples.

2.3. Preparation of standard solutions and quality control samples

Two stock standard solutions (1 mg/ml) of ketoconazole were prepared by dissolving the compound in methanol. One was used to make calibration standards, the other used for QC samples. For the validation work, these two stock standard solutions must agree within 5% of the LC–MS–MS response. Eight spiking standard solutions at concentrations of 0.100, 0.250, 0.500, 2.00, 5.00, 20.0, 40.0, 50.0 μg/ml were prepared by diluting the stock solution with methanol–water (1:1). The I.S. working solution was made at 1.00 μg/ml in methanol. All stock standard solutions and spiking/working standard solutions were stored in a refrigerator with temperature maintained at 2–8 °C. The stock standard solution of ketoconazole has been confirmed to be stable for at least 2 months with storage in a refrigerator.

The QC samples containing 60.0 (low-), 800 (medium-), and 7500 (high-) ng/ml ketoconazole in plasma were prepared. An over-the-curve (OTC) QC

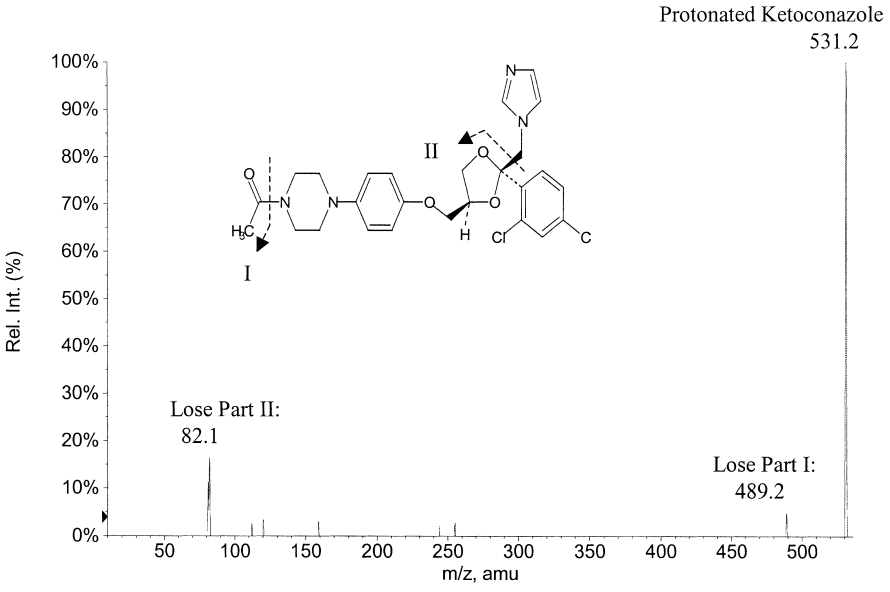

Fig. 2. Mass spectrum and fragmentation pathway of ketoconazole.

at 50 000 ng/ml in plasma, a lower limit of quantitation (LLOQ) QC at 20.0 ng/ml in plasma, and an ULOQ QC at 10 000 ng/ml in plasma were also prepared. All of these QC samples were made using the standard solutions from the second weighing. All pooled QCs were split into pre-labeled 0.5-ml polypropylene vials (approximately 0.3 ml sample per vial) and stored at approximately −20 °C.

2.4. Sample preparation

To make the calibration standard sample, 20.0 μl each of the eight spiking standard solutions was added into 0.100 ml control plasma in a 100×13 mm glass test tube with screw cap. For QC sample and clinical sample, 0.100 ml sample was directly aliquoted to the tube then 20.0 μl of methanol–water (1:1) added to make up the volume and matrix composition. A 25.0-μl volume of the I.S. working solution was added to each tube and vortex-mixed briefly. A 0.10-ml volume of 10% ammonium hydroxide was added to alkalinize the sample then 2.5 ml diethyl ether was used to extract the analyte and the I.S. by vortex-mixing for 3 min and centrifuging for 5 min at 2500 rpm and 15 °C. The organic portion was decanted to a pre-labeled clean conical glass tube after freezing the aqueous layer in a dry ice–acetone bath. The organic solvent was evaporated to complete dryness at 30 °C under a stream of nitrogen of 10 p.s.i. in a Turbo Vap water bath evaporator (1 p.s.i.=6894.76 Pa). The residue was then reconstituted in 0.400 ml of the mobile phase by vortexing for 1 min. A 10-μl aliquot of extract was injected into the LC–MS–MS system.

2.5. Validation

The precision and accuracy of the assay were evaluated by running three validation batches on 3 separate days. Each batch has one set of calibration standards and six replicates of QCs at low-, medium-, and high-concentration levels. One of the validation batches was also designed to examine the dilution integrity of partial volume. In such a batch six replicates of OTC QCs and extra six replicates of high-QCs (both using a partial volume 20.0-μl), treated with a fivefold dilution by blank plasma prior to extraction, were also processed. In one of the batches, six replicates of LLOQ QCs and ULOQ QCs were included as well. The short-term stability of the plasma QC samples was also tested in one validation batch. The short-term stability QC samples were obtained by allowing QC samples undergo three freeze–thaw cycles or sitting at room temperature for approximately 24 h (bench-top stability). One batch of the extracted samples was stored at room temperature (~22 °C) for approximately 24 h after being injected into the LC–MS–MS system then re-injected into the same system to check the storage and injector stability of the processed samples. The 2-month frozen plasma stability was tested using a calibration curve obtained from freshly prepared calibration standards to quantify the plasma QC concentrations after storing at approximately −20 °C for 2 months. For each analysis batch, the sample injection sequence was randomized through the entire run except the batch starting and ending with a calibration standard.

3. Results and discussion

3.1. Chromatography

The retention times of ketoconazole and R51012 on the BDS Hypersil C_{18} column vs. the percentage of ACN in the mobile phase are shown in Fig. 3. The content of ACN in the mobile phase varied from 20 to 90% (v/v). A complex and interesting retention phenomenon was noticed for both the analyte and the

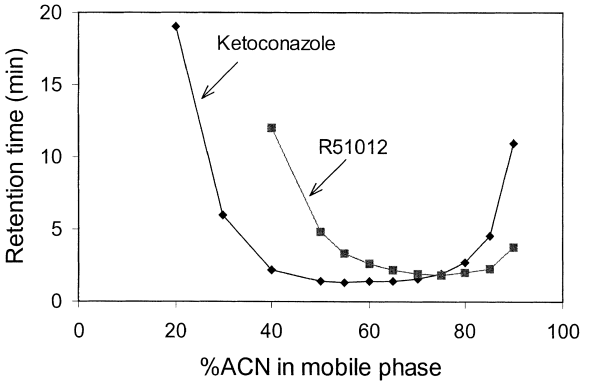

Fig. 3. Retention times of ketoconazole and R51012 vs. the percentage of acetonitrile in mobile phase.

I.S. Both showed that the retention time initially decreased and then increased with the increase of the content of ACN. This phenomenon could not be simply explained by either a reversed-phase or normal-phase retention mechanism although this was on the C_{18} column, a classically defined ''reversed-phase'' column. This observation reflected the complexity of interactions between mobile phase and stationary phase, between analyte and mobile phase, and between analyte and stationary phase, especially for those complex molecules with multiple ionizable functional groups such as ketoconazole and R51012 in this study. This also implied that on-column retention depends upon not only the column itself, but also the mobile phase and the analyte. Naidong et al. have discussed in detail the complexity of retention mechanism of compounds on analytical column and pointed out a C_{18} column should not be simply assumed to be reversed phase [24]. Due to the structural difference between the analyte and the I.S. in the current case, the % ACN of the mobile phase to achieve the minimum retention time for them was different. However, when a 75% ACN mobile phase was used, both ketoconazole and R51012 had similar retention time at ~1.8 min, see Fig. 3, and both compounds had the best peak shape and the maximum response, see Fig. 4. Having the similar retention times for the analyte and I.S. could minimize the potential matrix effects on the quantitation of biological samples if the co-elution does not cause significant suppression or enhancement from each other. In this case no co-elution effect was observed, thus the solution consisting of ACN–water–formic acid (75:25:1, v/v) was the best choice as the mobile phase.

The composition of an ideal injection solution was recommended to be ''weaker'' than the used mobile phase. The weaker or stronger solution is a relative concept, depending on the column-retention mechanism of the analyte. This requires a thorough understanding of the actual retention pattern for the specific compound on an analytical column [24]. Due to the complex retention behaviors for both ketoconazole and R51012, in this assay, the mobile phase, i.e., a mixture of acetonitrile–water–formic acid (75:25:1, v/v), was an reasonable choice for the reconstitution solvent. Fig. 5 shows representative mass chromatograms of the extracts from the human plasma blank, and control 0 (free of analyte), an LLOQ (20.0 ng/ml) sample, and an ULOQ (100 000 ng/ml) sample (last three samples spiked with I.S.) obtained on a BDS Hypersil 50×3 mm (5 μm) column under the chromatographic conditions described in Section 2.2.

3.2. Specificity

Six lots of blank plasma were tested for matrix effect and the assay specificity. For each lot of these plasma, a plasma blank (free of both analyte and I.S.), control 0 (plasma blank spiked with I.S. only), control 20 (spiked with 20.0 ng/ml analyte), and control 1000 (spiked with 1000 ng/ml analyte) were used to check interference and lot-to-lot matrix variation. The measured values and statistics are given in Table 1. For all of six plasma lots, the regions of the analyte and the I.S. were found to be free of interference. When these blank samples were spiked with ketoconazole at 20.0 ng/ml, the measured mean, relative standard deviation (RSD) and relative error (RE) were 21.0 ng/ml, 7.0%, and 5.0%, respectively. For these blank samples spiked with 1000 ng/ml of ketoconazole, the measured mean, RSD and RE were 994 ng/ml, 13.9% and −0.6%, respectively. The results showed that, except for lot 5 with a slight enhancement, all other five lots of plasma had no significant lot-to-lot matrix variation thus the method exhibited good specificity.

3.3. Sensitivity, linearity and recovery

The standard curve range was 20.0 to 10 000 ng/ml for ketoconazole calculated based on 0.1-ml plasma. Over the above curve range, the linear correlation coefficients were found to be better than or equal to 0.9985. This validated assay had an LLOQ of 20 ng/ml in plasma, which gave a signal-to-noise (S/N) ratio typically between 40 and 50. The detection limit to reach a S/N ratio of 3 was estimated to be ca. 2 ng/ml ketoconazole in plasma. In the present method, a dilution factor of 4 was made while reconstituting extract residue in a 0.4-ml mobile phase. If a further dilution was not applied, i.e., reconstituting residue in a 0.1-ml volume, a fourfold better sensitivity could be reasonably expected. The assay sensitivity could be even higher by

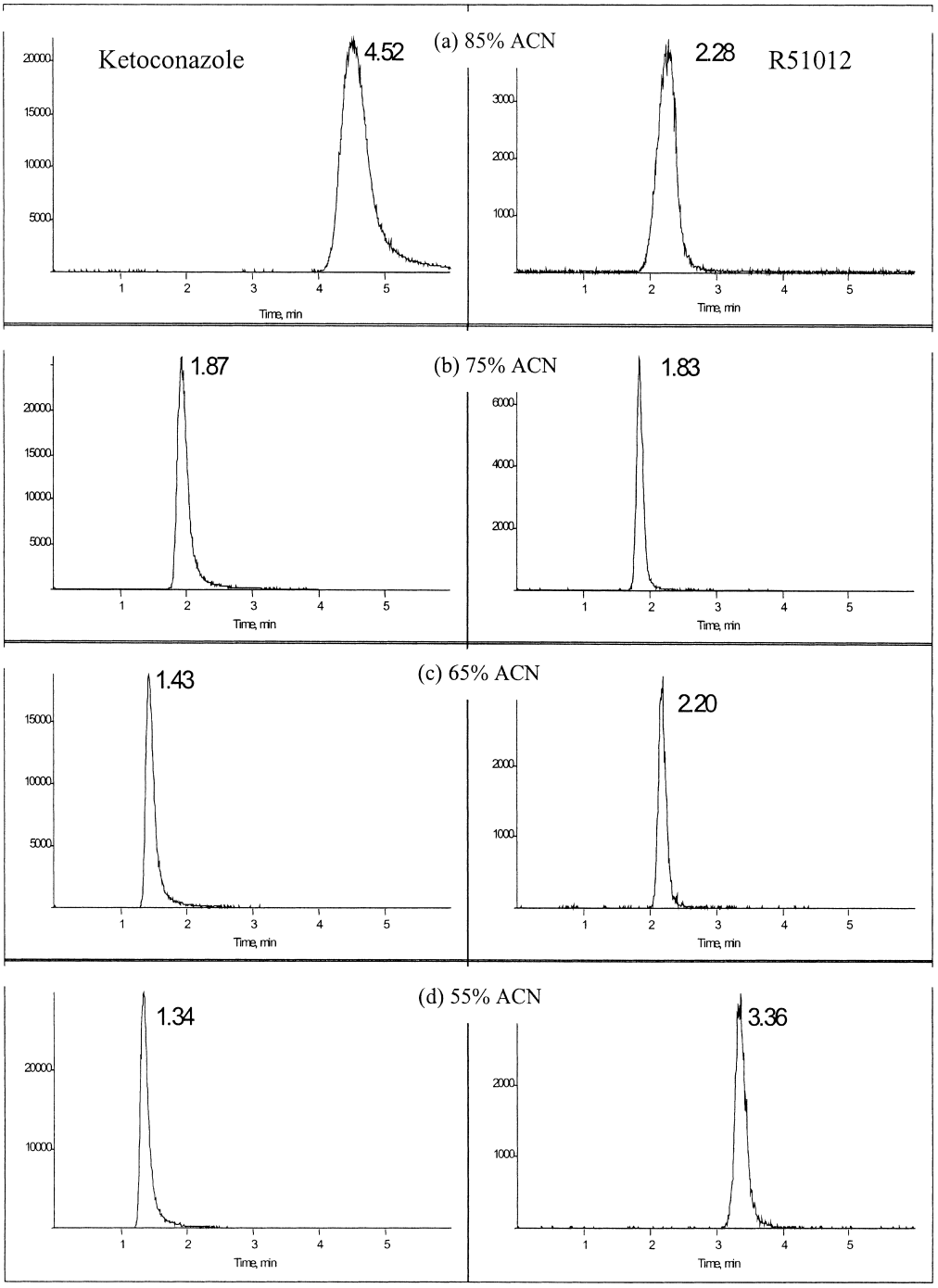

Fig. 4. Chromatograms of ketoconazole and R51012 obtained from the mobile phase containing different percentage of acetonitrile (ACN) (a) 85% ACN, (b) 75% ACN, (c) 65% ACN, and (d) 55% ACN (left panel: ketoconazole; right panel: R51012).

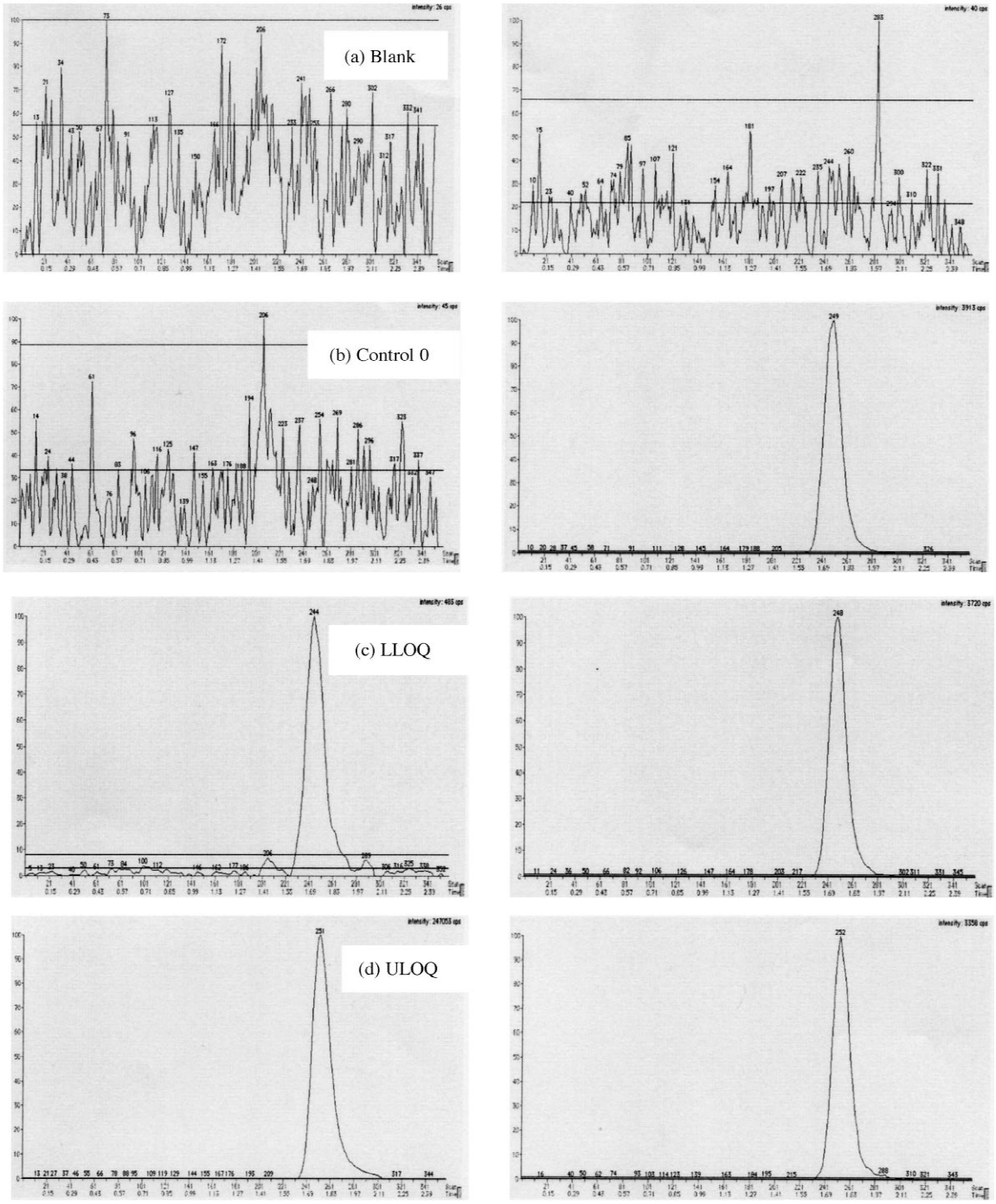

Fig. 5. Mass chromatograms of the extracted samples. Control plasma blank, (b) control 0 (spiked I.S. only), (c) LLOQ (20.0 ng/ml ketoconazole in plasma), and (d) ULOQ (10 000 ng/ml ketoconazole in plasma) (left panel: ketoconazole; right panel: R51012).

Table 1
Measured concentrations in spiked individual plasma lots

Lot No.	Spiked ketoconazole concentration	
	20.0 ng/ml	1000 ng/ml
1	20.3	931
2	21.6	929
3	20.7	1040
4	20.6	852
5	23.6	1246
6	19.3	964
Mean (ng/ml)	21.0	994
RSD (%)	7.0	13.9
RE (%)	5.0	−0.6

increasing injection volume. Thus this assay should be able to be modified to monitor 0.2–0.5 ng/ml ketoconazole in plasma without challenge. For the human study, the LLOQ of 20 ng/ml is sufficient. The extraction recovery of the present procedure was determined by comparing the peak areas of the analyte and I.S. extracted from each of low-, medium-, and high-QC levels with those of post-spiking compounds into blank plasma extracts at corresponding concentrations. The average recoveries were 102% for ketoconazole and 106% for the I.S. R51012. This indicated that this extraction procedure could quantitatively extract ketococonazole and the I.S. from plasma, and the I.S. R51012 tracked the ketoconazole extremely well.

3.4. Precision and accuracy

For all of three validation curves, the back calculation results for all calibration standards show ≤6.8% RSD and −3.8 to 6.0% RE, respectively (data not shown). The precision and accuracy for QC samples are given in Table 2. For the low-concentration (60.0 ng/ml) level QC, the precision and accuracy were 4.4% RSD and 1.4% RE for intra-day assay ($n=6$), and 5.8% RSD and −0.1% RE for inter-day assay ($n=18$). For the medium-concentration (800 ng/ml) level QC, the precision and accuracy were 2.2% RSD and −0.4% RE for intra-day assay ($n=6$), and 8.6% RSD and 0.9% RE for inter-day assay ($n=18$). For the high-concentration level QC, i.e., 7500 ng/ml, the precision and accuracy were found to be 3.3% RSD and −0.6% RE for intra-day assay ($n=6$), and 8.2% RSD and −1.4% RE for inter-day assay ($n=18$). The intra-day precision and accuracy were 3.9% RSD and 3.3% RE for LLOQ QC, and 4.0% RSD and −6.5% RE for ULOQ samples. These results demonstrated the present method had very good precision and accuracy.

Table 2
Intra-day and inter-day precision and accuracy of QC samples

	Ketoconazole in plasma (ng/ml)				
	LLOQ, 20.0	Low—60.0	Medium—800	High—7500	ULOQ, 10 000
Intra-day	*	61.7	765	7690	9740
($n=6$)	20.3	59.4	818	7290	9770
(except LLOQ, $n=5$)	20.2	58.3	792	7250	9020
	20.2	60.6	803	7510	9370
	20.5	59.3	805	7190	9360
	22.1	65.8	799	7780	8850
Mean (ng/ml)	20.7	60.9	797	7452	9352
RSD (%)	3.9	4.4	2.2	3.3	4.0
RE (%)	3.3	1.4	−0.4	−0.6	−6.5
Inter-day ($n=18$)					
Mean (ng/ml)	–	59.9	807	7398	–
RSD (%)	–	5.8	8.6	8.2	–
RE (%)	–	−0.1	0.9	−1.4	–

*Data excluded because of poor injection.

Table 3
Dilution integrity of plasma samples: partial volume with a fivefold dilution ($n=6$)

	Ketoconazole in plasma (μg/ml)	
	High QC, 7.50	OTC QC, 50.0
	8.79	52.4
	8.85	54.3
	8.39	51.9
	7.93	53.8
	8.35	48.9
	7.41	51.7
Mean (ng/ml)	8.29	52.2
RSD (%)	6.6	3.7
RE (%)	10.5	4.3

3.5. Dilution integrity, stability and ruggedness

A fivefold dilution for OTC QC sample and high-concentration QC sample by matrix blank prior to extraction was used to evaluate dilution integrity. Six replicates of partial volume OTC QC and high-QC samples were processed in one of the validation batches. The data are shown in Table 3. The results indicated that the taking partial volume and diluting with matrix blank did not give significant deviation for the analytical data.

The stability experiments aimed at testing all possible conditions that the samples might experience during the sample shipping and handling such as freezing–thawing and a short storage at room temperature (bench-top) and during analysis such as extracted samples sitting in sample tray or refrigerator, etc. These were performed as described in Section 2.5. All stability results are summarized in Table 4. Three freeze–thaw cycles and 24-h room temperature storage for QC samples had no substantial effect on the results. Keeping extracts at room temperature for approximately 24 h prior to injection did not affect the quantitative determination of ketoconazole in samples.

Table 4
Stability data

	Ketoconazole in plasma (ng/ml)		
	Nominal QC		
	60.0	800	7500
Three freeze–thaw cycles ($n=6$)			
Mean (ng/ml)	59.8	798	7622
RSD (%)	1.8	4.1	2.3
Accuracy (%)	99.7	99.8	101.6
Bench-top ambient ~24 h ($n=6$)			
Mean (ng/ml)	62.1	807	7528
RSD (%)	4.5	3.0	3.9
Accuracy (%)	103.5	100.9	100.4
Extracts stored ambient ~24 h ($n=6$)			
Mean (ng/ml)	58.6	754	7292
RSD (%)	1.2	5.1	4.9
Accuracy (%)	97.7	94.3	97.2
Two-month QC samples storage at −20 °C ($n=3$)			
Mean (ng/ml)	64.1	760	7683
RSD (%)	6.1	9.2	11.5
Accuracy (%)	106.9	95.0	102.4

The 2-month storage stability of QC samples has also been tested and the data were included in Table 4. The results show a better than 11.5% RSD ($n=3$ for each level QC) and 95.0 to 106.9% accuracy, indicating that the QC samples were stable for at least 2 months if storing frozen at approximately −20 °C.

One set of the validation samples including calibration standards and six replicates of each level regular QC samples were re-analyzed on another LC–MS–MS system using another analytical column. The statistics of the data obtained from the another system were the standard curve linearity $r^2=0.9979$ (0.9989), mean 59.6 ng/ml, 4.7% RSD and −0.7% RE (mean 57.7 ng/ml, 4.5% RSD and −3.9% RE) for low-level QC (nominal value 60.0 ng/ml); mean 783 ng/ml, 4.4% RSD and −2.2% RE (mean 770 ng/ml, 3.5% RSD and −3.8% RE) for medium-level QC (nominal value 800 ng/ml); mean 7058 ng/ml, 5.9% RSD and −5.9% RE (mean 7032 ng/ml, 3.9% RSD and −6.2% RE) for high-level QC (nominal value 7500 ng/ml), respectively. The data shown in parentheses were obtained from the original system. Both sets of results were in excellent agreement. It was also noted that no significant back-pressure increase or other evidences on column disorder were noticed after at least one thousand injections into the same analytical column. These observations indicated this assay was rugged.

3.6. Application

The validated method has been successfully used to monitor the ketoconazole concentration in the human plasma samples from PK study for a pharmaceutical company. Since we did not own those data, an independent in-house study was carried out by a single oral dose of 5-mg ketoconazole to monkey. The above method has been modified and cross-validated for the monkey study with an LLOQ of 1 ng/ml by injecting 20 μl based on a 0.1 ml sample size and reconstituting in 0.1 ml solvent. A representative PK profile for the monkey study is shown in Fig. 6. The PK parameters were found to be $C_{max}=180$ ng/ml at $T_{max}=0.5$ h, $AUC_{(0-24)}=236$ ng/ml h and $AUC_{(0-\infty)}=257$ ng/ml h. This PK curve exhibited a two-phase profile with $T_{1/2}=0.78$ h in the α-phase and $T_{1/2}=14.3$ h in the β-phase.

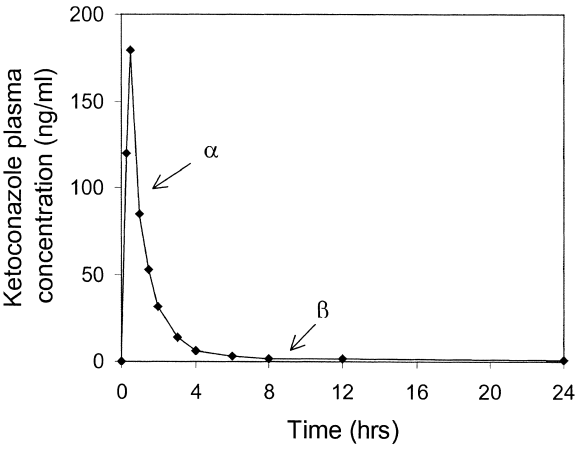

Fig. 6. Pharmacokinetic profile of a monkey study with a single oral dose of 5 mg ketoconazole.

4. Conclusion

A simple and rapid LC–MS–MS method for the determination of ketoconazole in human plasma was developed and validated. This method used a simple one-step liquid–liquid extraction and a C_{18} column coupled with MS–MS for separation and detection. The results showed good precision and accuracy. The validated assay used a 0.1-ml plasma sample and the standard curve range was 20.0 to 10 000 ng/ml in human plasma, which covered the concentration range in clinical samples. The described method optimized chromatographic conditions through the understanding of different retention behaviors of analyte and I.S. Due to the high sensitivity of MS–MS detection, the method could be modified for microsample analysis or lower concentration level. The present method has been successfully applied to support PK studies.

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