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Liquid/liquid extraction using 96-well plate format in conjunction with hydrophilic interaction liquid chromatography–tandem mass spectrometry method for the analysis of fluconazole in human plasma

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Abstract

A bioanalytical method using automated sample transferring, automated liquid/liquid extraction (LLE) and hydrophilic interaction liquid chromatography–tandem mass spectrometry was developed for the determination of fluconazole in human plasma. Samples of 0.05 ml were transferred into 96-well plate using automatic liquid handler (Multiprobe™ II). Automated LLE was carried out on a 96-channel programmable liquid handling workstation (Quadra™ 96) using methyl-*tetra* butyl ether as the extraction solvent. The extract was evaporated to dryness, reconstituted, and injected onto a silica column using an aqueous–organic mobile phase. The chromatographic run time was 2.0 min per injection, with retention times of 1.47 and 1.44 min for fluconazole and internal standard (IS) ritonavir, respectively. The detection was by monitoring fluconazole at m/z 307 → 238 and IS at m/z 721 → 296, respectively. The standard curve range was 0.5–100 ng ml^{-1}. The inter-day precision and accuracy of the quality control samples were < 7.1% relative standard deviation and < 2.2% relative error.

Keywords: Fluconazole; HILIC-MS/MS; Human plasma; 96-Well liquid/liquid extraction

1. Introduction

Fluconazole (2,4-difluoro-α-α[1]-bis(1H-1,2,4-triazol-1-ylmethyl)benzyl alcohol, see Fig. 1 for chemical structure), a highly selective inhibitor of fungal cytochrome P-450 sterol C-14 alpha-demethylation, has been widely used for the prevention and treatment of broad range of fungal infections that predominantly affect immuno-compromised patients. Mammalian cell demethylation is much less sensitive to fluconazole inhibition. In normal volunteers, fluconazole is cleared primarily by renal excretion, with approximately 80% of the administered dose appearing in the urine as unchanged drug [1]. Clinical pharmacokinetics of fluconazole indicate that fluconazole is effective at

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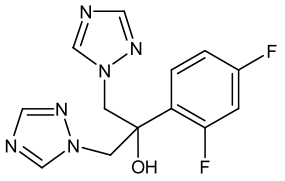
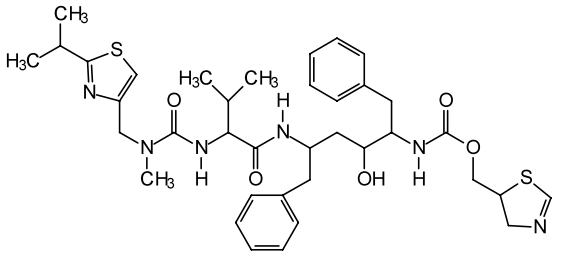


Fluconazole



Ritonavir

Fig. 1. Chemical structures of fluconazole and IS ritonavir.

a wide range of body sites because of its large distribution volume, the long half-life and mean residence times, combined with a rapid absorption after oral administration [1–3]. Because fluconazole is an inhibitor of certain human cytochrome P-450 isozymes, drug–drug interaction studies involving fluconazole have been extensively reported in Refs. [4–12]. Numerous analytical methods, including bioassay [13], gas chromatography [14], liquid chromatography (LC) with ultraviolet detection [15–23], and LC with tandem mass spectrometry (LC-MS/MS) [24,25] have appeared in literature to analyze fluconazole in biological fluids. Methods using non-mass spectrometric detection are tedious and may not have the desired selectivity. The published LC-MS/MS methods provided the additional selectivity offered by the tandem mass spectrometers. However, lack of automation for these LC-MS/MS methods make them less desirable for analyzing large quantity of samples from clinical studies.

In this paper, we present a highly automated hydrophilic interaction LC-MS/MS (HILIC-MS/MS) method for the assay of fluconazole in human plasma, which can be routinely used to support drug–drug interaction studies. Particular effort was made to automate the sample preparation step. As a direct result of the short analysis times offered by LC-MS/MS methods, sample preparation has become the rate-limiting step [26]. Much effort has been devoted to automate the sample preparation step by using 96-well plate format including liquid/liquid extraction (LLE) [27,28]. The method described here also utilized automated sample transferring and LLE in 96-well plate format.

2. Experimental

2.1. Chemicals and reagents

Fluconazole (purity 100%) was kindly supplied by Pfizer (New York, NY), and internal standard (IS) ritonavir (purity 100%) was supplied by Custom Synthesis Services (Madison, WI). Upon receipt, these reference standards were stored refrigerated. Acetonitrile, methanol, methyl-*tetra* butyl ether (MTBE), ammonium hydroxide, and water were of LC grade and were from Fisher Scientific (St. Louis, MO). Trifluoroacetic acid (TFA) of LC grade, in 1-ml ampoule, was from Sigma (St. Louis, MO). Blank human plasma with potassium edentate (K_3EDTA) as anticoagulant was from Biochemed (Winchester, VA) and was stored in a freezer at -20 °C.

2.2. Calibration standards and quality control samples

Standards and quality controls (QCs) were made from two separate stock solutions (1 mg ml^{-1} in methanol–water 1:1, v/v) of fluconazole. For the validation work, these two stock standard solutions must agree within 5% of the HILIC-MS/MS response. The stock solutions were stored in polypropylene tubes with screw caps and were stable for at least 1 month when kept in a refrigerator at 2–8 °C. Pooled calibration standards at concentrations of 0.50, 1.00, 2.50, 7.50, 25.0, 50.0, 80, and 100 ng ml^{-1} were prepared in blank plasma pool made by combining 6 lots of blank plasma. QCs at levels of 1.50, 10.0, and 70.0 ng ml^{-1} were prepared for the determination of intraday and interday accuracy and precision.

Over the curve QCs were prepared at 140 ng ml^{-1} and low limit of quantitation (LLOQ) QCs were prepared at 0.50 ng ml^{-1}. The volumes of the spiking solutions were always kept below 1% of the plasma volumes. All standards and QCs were aliquoted into pre-labeled 0.5-ml polypropylene vials and stored frozen at −20 °C.

2.3. HILIC-MS/MS

The HILIC-MS/MS system consisted of a Shimadzu HPLC system (Kyoto, Japan) and a PE Sciex API 3000 tandem mass spectrometer (Concord, Ont., Canada) with (+) ESI. The analytical column, Betasil silica of 5 μm, 50 × 3.0 mm I.D., was from Keystone Scientific (Bellefonte, PA, USA). An Inertsil ODS C18 column of 5 μm, 50 × 3.0 mm I.D., from Keystone Scientific was used to compare the fluconazole sensitivity on silica and C18 columns. The columns were maintained at ambient temperature. The final mobile phase was acetonitrile–water–TFA (90:10:0.05, v/v/v) on the silica column. The injection volume was 20 μl; run time was 2.0 min; flow rate was 0.5 ml min^{-1}. Autosampler carry-over was determined by injecting the highest calibration standard then an extracted blank sample. No carry-over was observed, as indicated by the lack of either fluconazole or IS peak in the blank sample. The background noise in the blank sample was also not elevated. Without any column-regeneration, the silica column could be used for at least 500 injections of the extracted samples. The column performance throughout the study was monitored by measuring the retention time (t_R), peak symmetry factor (B/A) at the 10% peak height where B and A were the distances after and before the peak center respectively, and the plate count (N) as 5.54 $(t_R/W_{0.5})^2$ where $W_{0.5}$ was the peak width at the half of the peak height.

Sensitivity of the multiple reaction monitoring (MRM) was optimized by testing with an infusion of 0.1 μg ml^{-1} fluconazole in a mixture of acetonitrile and water (1:1, v/v). The ionspray needle was maintained at 5 kV. The turbo gas temperature was 400 °C and the auxiliary gas flow was 8.0 l min^{-1}. Nebulizing gas, curtain gas, and collision gas flows were at instrument settings of 8, 8, and 5, respectively. The declustering potential and focusing potential were at 46 and 200 V, respectively. The mass spectrometer was operated under MRM mode with a collision energy of 24 eV. The transitions (precursor to product) monitored were m/z 307 → 238 for fluconazole, and 721 → 296 for IS. The dwell time was 300 ms for each transition. Both quadrupoles were maintained at unit resolution.

Chromatograms were integrated using the ANALYST version 1.1 software. A weighted 1/concentration^2 linear regression was used to generate calibration curves from standards and calculate the concentrations of QC samples.

2.4. Sample preparation

Samples were briefly vortex-mixed and centrifuged at 3000 rpm for 5 min at room temperature on a Beckman Coulter J2-HS centrifuge (Fullerton, CA). Fifty microliter were then transferred from vials into 1-ml 96-well deep well plates from Porvair Sciences (Shepperton, UK) by the Packard Multiprobe™ II robotic liquid handler (Meriden, CT) controlled by the WINPREP™ software. Between two consecutive pipetting, the Multiprobe needles were washed with water, 0.5% (v/v) TFA in acetonitrile, and water. Carry-over of the Multiprobe needles was not observed. No fluconazole peak was detected in the four blank samples after pipetting four highest standards. Each individual needle was calibrated for accuracy and precision by pipetting water into pre-weighted tubes. These tubes were weighted again to calculate the actual pipetting volume at both 10 and 50 μl. Twenty microliter of IS spiking solution (250 ng ml^{-1} in water–methanol, 1:1) were then added to all samples except blanks to which 20 μl water–methanol (1:1) was added. Cover the plate with a dimpled sealing mat from Porvair Sciences and vortex-mix the plate at low speed for approximately 30 s. After removing the dimpled cover, the sample plate was then brought to Tomtec Quadra™ 96–320 robot (Hamden, CT). Add 50 μl of 2% (v/v) ammonium hydroxide aqueous solution and cover the plate with a dimpled sealing mat and vortex-mix the 96-well plate at low speed for approximately 30 s. Remove the dimpled cover.

Using the Tomtec, 0.450 ml of MTBE was added and the plate was covered tightly with a dimpled cover. Vortex-mix the plate at high speed for approximately 2 min. Centrifuge the plate at 3000 rpm for approximately 5 min at room temperature. Carefully remove the dimpled cover. Using the Tomtec, transfer approximately 0.35 ml of MTBE layer to a new 1-ml 96-well deep well plate. The MTBE extracts in collection plate were evaporated to dryness under a gentle stream of nitrogen at about 30 °C using a TurboVap™ 96 concentrator (Zymark, Hopkinton, MA) and reconstituted with 200 μl of 0.05% (v/v) TFA in acetonitrile using the Tomtec robot. The advantage of using a reconstitution solution with elution strength weaker than the mobile phase has been discussed [29]. The plate was then heat-sealed with a Uniseal™ film (Whatman, Clifton, NJ). To assist the reconstitution, the plate was vortex-mixed at low speed for approximately 2 min.

2.5. Validation of the HILIC-MS/MS method

The method was validated by three consecutive analytical curves on 3 separate days. Each calibration curve contained a single set of calibration standards and six replicates of QCs at each concentration level. One calibration curve also included LLOQ QCs (0.50 ng ml^{-1}), and over the curve QCs (140 ng ml^{-1}), which were diluted 5-fold with control blank plasma prior to analysis. Each curve also contained other test samples such as processing and storage stability samples. Calibration standards, QCs and other test samples were randomized through the curve. An extracted blank sample was always placed after the ULOQ standard (upper limit of quantitation) to determine carry-over of the HILIC-MS/MS system. One curve contained 96 samples to simulate the length of clinical sample analysis.

The method specificity was evaluated by screening six lots of blank plasma. These lots were spiked with fluconazole at 0.00, 1.50, and 70.0 ng ml^{-1}. The spiked samples were extracted and analyzed to confirm lack of interference and absence of lot-to-lot variation. The matrix suppression was determined by comparing the peak areas of analyte spiked post extraction with those in unextracted solutions at corresponding concentrations.

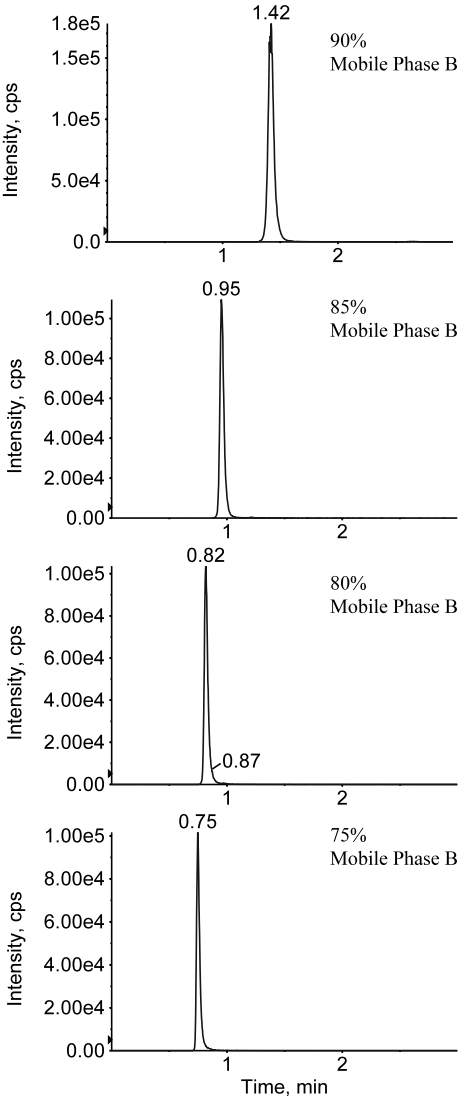


Fig. 2. Chromatograms of fluconazole on a silica column with aqueous–organic mobile phases. Column, Betasil silica 50 × 3 mm, I.D., 5 μm; mobile phase *A*, water–TFA (100:0.05, v/v); mobile phase *B*, acetonitrile–TFA (100:0.05, v/v); flow rate, 0.5 ml min^{-1}; injection volume, 5 μl of 100 ng ml^{-1} of fluconazole.

Analyte stability was tested by subjecting QCs through multiple freeze–thaw cycles, and on the bench at room temperature. Post-extraction analyte stability was also determined.

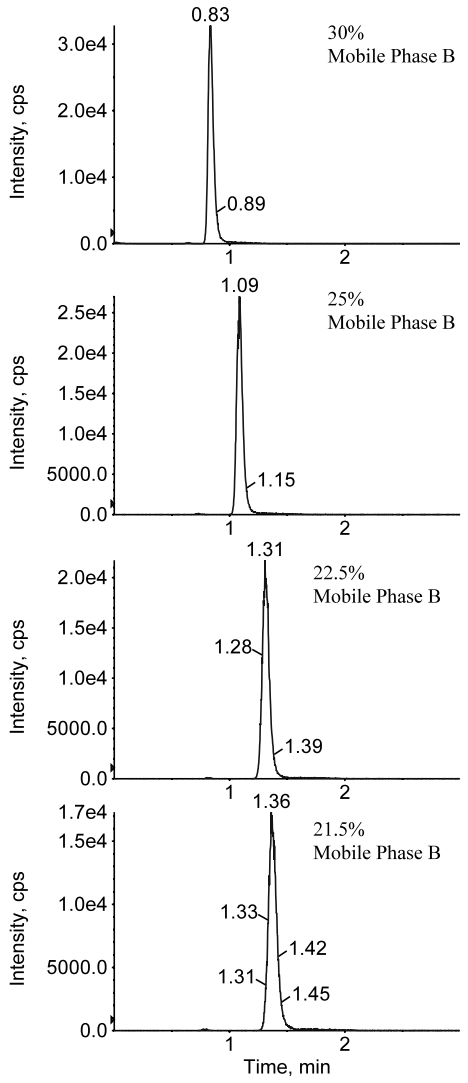


Fig. 3. Chromatograms of fluconazole on a C18 column with aqueous–organic mobile phases. Column, Inertsil C18 50 × 3 mm, I.D., 5 μm; mobile phase *A*, water–TFA (100:0.05, v/v): mobile phase *B*, acetonitrile–TFA (100:0.05, v/v); flow rate, 0.5 ml min^{-1}; injection volume, 5 μl of 100 ng ml^{-1} of fluconazole.

Extraction recovery was determined by comparing the peak areas of the analyte extracted from plasma with those of post-extraction spiked plasma blanks at corresponding concentrations. Since during the extraction only 0.35 ml out of total 0.45 ml of MTBE was transferred, the extraction recovery was therefore corrected for the volume change.

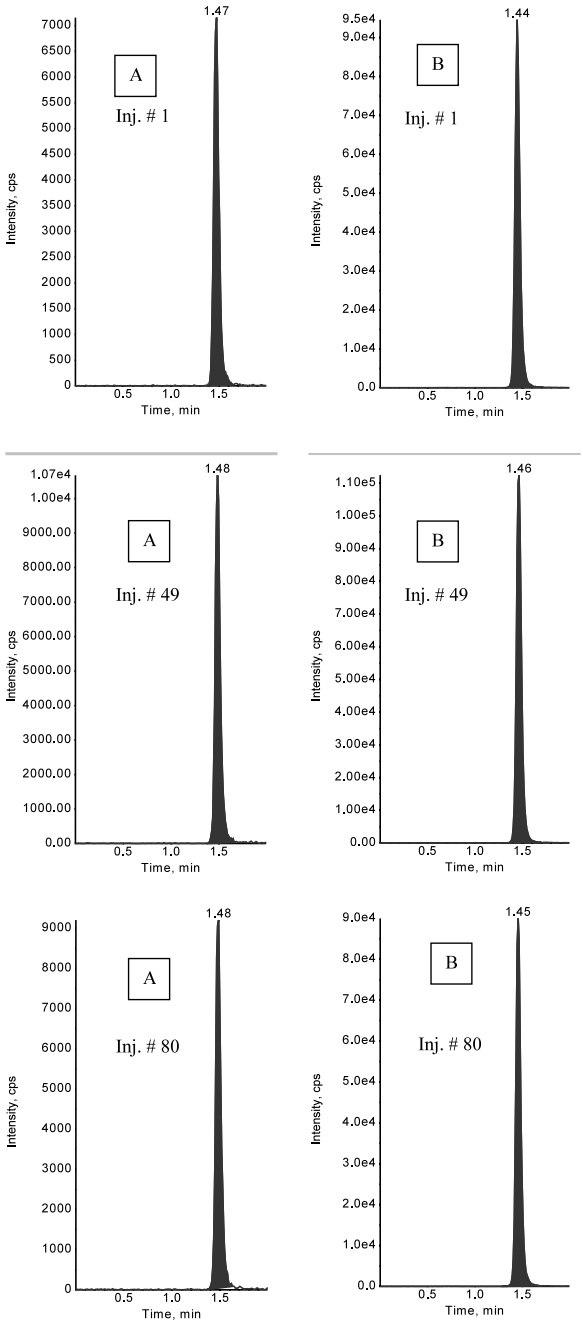


Fig. 4. Silica column stability: selected ion current traces of fluconazole and IS of the human plasma spiked with (A) 10 ng ml^{-1} fluconazole; (B) IS.

The method ruggedness was evaluated by injecting an extracted curve onto multiple LC-MS-MS instruments using analytical columns from different lots.

3. Results and discussion

3.1. HILIC-MS/MS

The selection of silica column and aqueous–organic mobile phase for quantitative analysis of fluconazole was based on our previous experiences for analyzing other polar compounds in biological fluids [30–39]. Bare silica columns operated with low aqueous–high organic mobile phases are viable means of analyzing polar compounds in biological fluid. Mobile phases containing highly organic solutions would lead to favorable spraying conditions at the LC-MS interface necessary for adequate sensitivity [40]. Even though TFA was reported to suppress electrospray signals due to its ion-pairing in the gas phase with the analyte ion [41], the gain of sensitivity by using higher organic content was so large that the overall sensitivity was still enhanced. Figs. 2 and 3 show the chromatograms of fluconazole on a silica column and on a C18 column respectively with mobile phases of different composition. On the silica column, when the TFA concentration was kept constant at 0.05%, increasing acetonitrile concentration in the mobile phases not only increased fluconazole on column retention, as predicted from the HILIC mechanism, but also enhanced its signal intensity due to the more favorable spraying condition with a mobile phase of higher organic content. On the C18 column, with the increase of acetonitrile the retention for fluconazole decreases, as predicted by the reversed-phase mechanism. When similar on column retention was achieved for fluconazole (90% acetonitrile on silica column and 21.5% acetonitrile on C18 column), the signal intensity on the silica column is about 10-fold higher than that on the C18 column. This type of sensitivity improvement on silica columns over reversed phase columns have also been observed for other polar compounds such as nicotine, cotinine, albuterol, bamethan [30]; fentanyl [33]; and clonidine, ritonavir, naltrexone, loratadine [38].

The silica column demonstrated excellent stability as shown in Fig. 4, indicated by very stable t_R and unchanged peak shape for both fluconazole and IS. After about 500 injections of extracted samples, the fluconazole retention changed from 1.47 to 1.42 min. The plate count (N) for fluconazole was changed from 3450 to 3380. The peak symmetry remained to be 1.1. This is similar to what we observed for other compounds [34].

3.2. Automation

The automation strategy involved in this method is to separate the sample transfer step from the extraction step. The use of a single robotic liquid handler with either 4 or 8 tips (i.e. Packard Multiprobe™ II) was ideal for the sample transfer. Before use, each of the four tips on the Multiprobe™ II was checked for accuracy and precision at 10 and 50 μl by pipetting and weighing water. The relative standard deviation (RSD) and relative error (RE) values were below 5% for each tip. Furthermore, the good precision for pipetting plasma samples was reflected by the very low RSD values for the calibration standards and QCs. The good accuracy for pipetting plasma samples was demonstrated by the very low RE values for QCs (70 and 140 ng ml^{-1}) which were diluted 5-fold ($10 \rightarrow 50$ μl) that by the Multiprobe™ II.

Packard Multiprobe™ II has the flexibility of pipetting samples from tubes to 96-well plate or to another set of tubes. In theory, Multiprobe™ II could also be used to transfer organic solvents like MTBE but the throughput would be seriously hampered due to the limited number of tips. Extraction was thus carried out in an essentially serial fashion. The relative long time taken for the reagent addition further contributed to potential solvent (MTBE) evaporation, leading to inconsistent extraction. On the other hand, Tomtec Quadra™ 96 workstation is equipped with 96 tips and is capable of extracting 96 samples simultaneously. However, the Quadra™ 96 is usually neither accurate nor flexible enough to transfer samples or to add IS. In the current method, the Multiprobe™ II was programmed to

Table 1
Throughput comparison for LLE of 96 samples: manual vs. automated

	Manual	Automated
Tube labeling	**20 min**	None
Centrifugation	None	**20 min**
Sample aliquoting	**75 min**	30 min
Extraction	**100 min**	**20 min**
Evaporation	15 min	15 min
Reconstitution and transfer	**30 min**	**10 min**
Total time	4 h	1 h 35 min
Analyst time	**3 h 45 min**	**50 min**

The time with bold line indicates the presence of analyst is required.

aliquot samples from individual tubes to 96-well deep well plates and to add IS. The plate was then brought to Quadra™ 96 for the LLE sample cleanup. This separation of the two steps not only maximized the ability of Multiprobe™ II's ability to accurately and flexibly transfer samples and IS, but also fully utilized the Quadra™ 96's parallel processing capability. Table 1 compares the throughput for manual and automated process of 96 samples. The total extraction time was reduced from 4 h for the manual operation to 1 h 35 min for the automated operation. The major time saving of using automation is at the sample aliquoting and extraction steps. To prevent the Multiprobe needle clogging, a sample centrifugation step is needed for the automated sample aliquoting. This step is usually unnecessary for the manual aliquoting. For the LLE using 96-well plate format, steps of covering the plates with dimpled cover, vortex-mixing, centrifuging the plate, and removing dimpled cover were used. Care must be exercised to prevent potential contamination across the wells. At our hands, the dimpled sealing mat from Porvair Sciences worked remarkably well and no cross contamination was observed. The time involving analyst's presence was dramatically reduced from 3 h 45 min for the manual operation to 50 min for the automated operation. This freed analyst to do other tasks such as reviewing data, documenting results, planning experiments etc. This observation of the labor saving is similar to what was observed by Jemal et al. for 96-well solid phase extraction [42].

3.3. Validation results

Six lots of blank control plasma were tested for matrix interference. Matrix effects from co-eluting endogenous components in biological fluids have been well documented in the literature to compromise the reproducibility and accuracy of the analysis [43–45]. The regions of the analyte and IS peaks were free from interference. When the samples were spiked with fluconazole at low QC concentration, which is 1.50 ng ml^{-1}, the RSD

Table 2
Matrix lot-to-lot reproducibility

Matrix lot #	Theoretical concentration (ng ml^{-1})			HILIC-MS/MS peak area					
	0.00	1.50	70.0	0.00		1.50		70.0	
	Measured concentration (ng ml^{-1})			Flu	IS	Flu	IS	Flu	IS
1	0.00	1.45	70.5	0	161727	2329	155956	116937	169509
2	0.00	1.45	77.6	0	165158	2327	155628	129012	169837
3	0.00	1.39	75.1	0	157731	2204	152713	121081	164901
4	0.00	1.48	71.1	0	170250	2458	160791	122300	174313
5	0.00	1.63	70.2	0	160506	2639	157790	118334	172287
6	0.00	1.58	68.8	0	163835	2644	163171	122029	181241
Mean	0.00	1.50	72.3	0	163201	2434	157675	121616	172014
RSD (%)	N/A	6.0	4.4	N/A	2.6	7.4	2.4	3.5	3.2
RE (%)	N/A	0.0	+3.3	N/A	N/A	N/A	N/A	N/A	N/A

Flu, fluconazole; IS, internal standard; RSD, relative standard deviation; RE, relative error calculated as (measured value/nominal value) × 100−100%; N/A, not applicable.

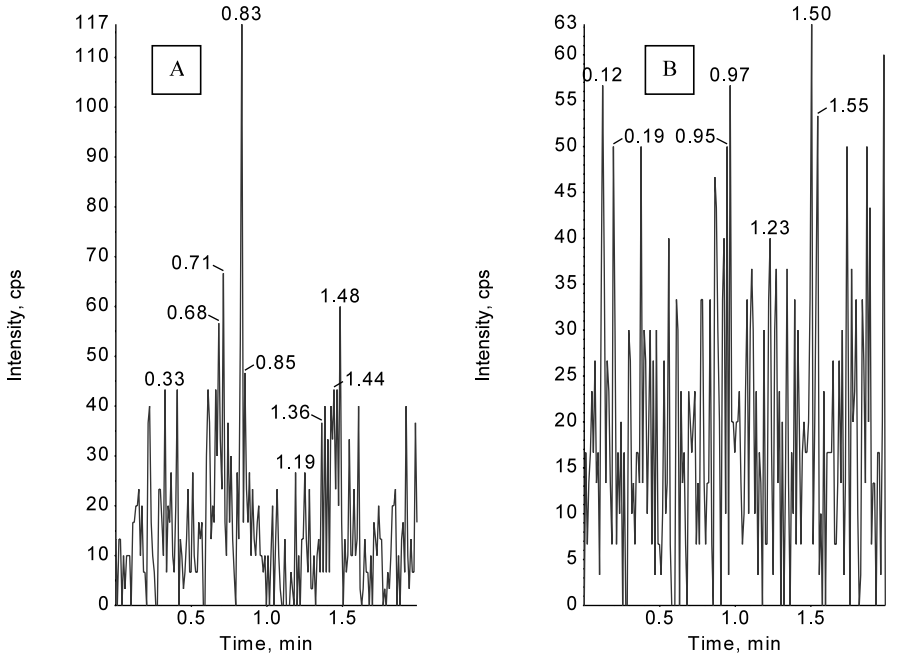


Fig. 5. Chromatogram of a blank plasma sample. (A) Fluconazole channel, 307→238; (B) IS channel, 721→296.

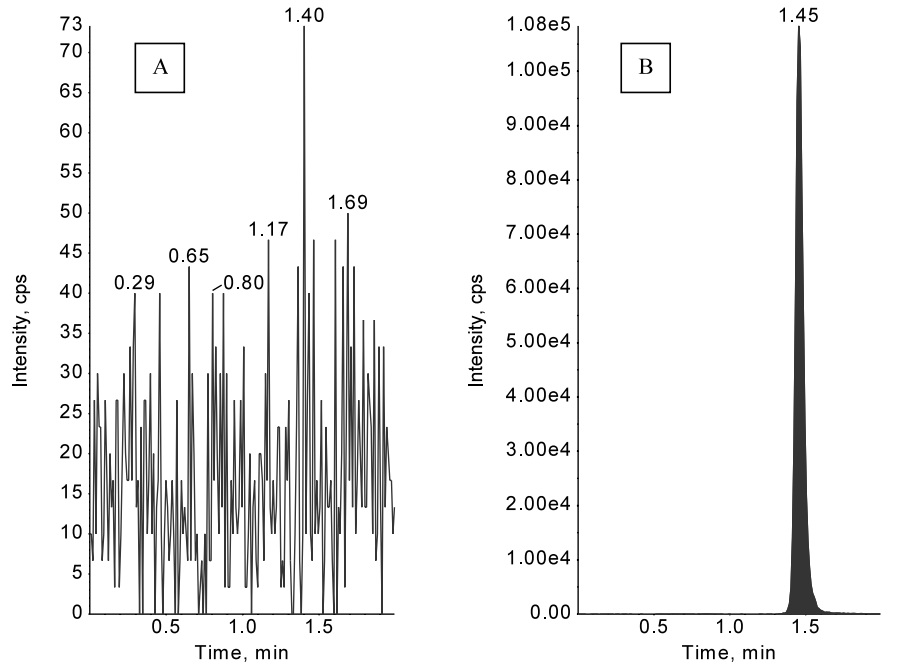


Fig. 6. Chromatogram of a blank plasma sample spiked with only IS. (A) Fluconazole channel, 307→238; (B) IS channel, 721→296.

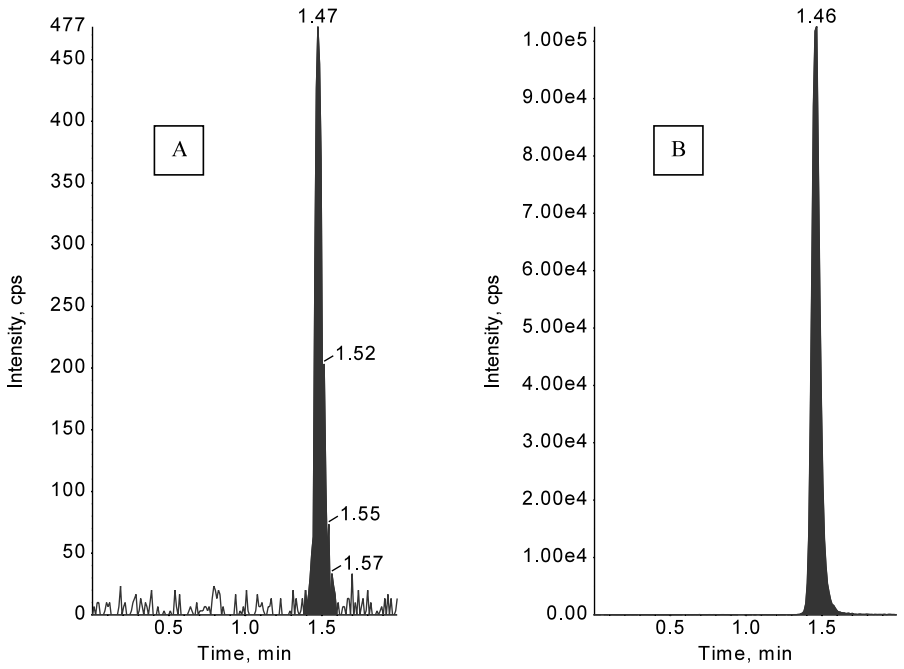


Fig. 7. Chromatogram of a blank plasma sample spiked with fluconazole at LLOQ (0.5 ng ml^{-1}) and IS. (A) Fluconazole channel, 307 → 238; (B) IS channel, 721 → 296.

and RE were 6.0 and 0.0%, respectively as shown in Table 2. For the samples spiked with fluconazole at high QC concentration that is 70 ng ml^{-1}, the RSDs and REs were 4.4 and +3.3%, respectively. These tight RSD and RE values indicate no significant lot-to-lot variation in matrix effects. The absolute magnitude of matrix suppression was determined by comparing peak areas of post-extraction spiked samples with those from unextracted analyte at the same concentration. For fluconazole, the matrix suppression is $<10\%$ ($<$ 10% of the analyte signal was suppressed) for both 2.5 and 80 ng ml^{-1} samples. This suppression is insignificant and could be well within the measurement errors. However, for IS that is ritonavir, the suppression is more severe. About 25% of the IS signal is suppressed. It should be noted that the chromatographic t_R for IS and for fluconazole is almost identical as shown in Fig. 4. The apparent difference for the matrix suppression for these two compounds is probably due to their different sensitivity coefficients, which dictate the magnitude of the suppression. The detailed theoretical consideration had been described by Kebarle and Ho [46] as well as Zhou et al., [47]. Despite of the relative high matrix suppression for the IS, the variability of the matrix suppression among the tested plasma lots was very low. The results were also shown in Table 2. Consistent peak areas for both fluconazole and IS were obtained for all six lots of plasma. Suppression caused from fluconazole to IS and vice verse was not observed. As demonstrated here, the use of a non-stable isotope labeled IS necessitated the careful examination of matrix effects and recovery.

Initially, other compounds such as ketoconazole were evaluated for potential use as the IS. However, it was soon discovered that none of these compounds tracked fluconazole well in the sample extraction or chromatographic elution. Finally, ritonavir was chosen because it worked well tracking fluconazole despite their apparent difference in chemical structure.

Since fluconazole is cleared primarily by renal excretion, with approximately 80% of the administered dose appearing in the urine as unchanged drug and only 11% of the dose is excreted in the urine as metabolites, the potential matrix effects

Table 3
Precision and accuracy of calibration standards ($n = 3$)

	(ng ml^{-1})								
	0.500	1.00	2.50	7.50	25.0	50.0	80.0	100	r^2
Batch 1	0.510	0.985	2.30	7.97	24.8	50.3	79.3	102	0.9989
Batch 2	0.481	1.09	2.36	7.78	27.3	46.0	81.8	93.1	0.9968
Batch 3	0.499	0.998	2.46	8.04	27.9	45.4	77.5	95.4	0.9972
Mean	0.497	1.02	2.37	7.93	26.7	47.2	79.5	96.8	0.9976
RSD (%)	2.9	5.6	3.4	1.7	6.2	5.7	2.7	4.8	0.1
RE (%)	−0.7	+2.4	−5.1	+5.7	+6.7	−5.5	−0.6	−3.2	N/A

Table 4
Precision and accuracy of QC samples

	Intraday ($n = 6$) (ng ml^{-1})						Interday ($n = 18$) (ng ml^{-1})		
	0.500	1.50	10.0	70.0	70.0[a]	140[a]	1.50	10.0	70.0
Mean	0.430	1.56	10.6	73.0	74.9	154	1.51	10.2	70.7
RSD (%)	5.8	6.5	5.4	7.5	4.6	4.5	7.1	4.9	6.3
RE (%)	−14	+3.7	+5.6	+4.2	+7.0	+10.0	+0.7	+2.2	+1.0

[a] Samples were diluted 5-fold with blank plasma prior to analysis.

that could be caused by metabolites were deemed minimal.

Representative chromatograms of a blank matrix, a control zero (blank plasma spiked with IS only), and a LLOQ (0.5 ng ml^{-1}) are shown in Figs. 5–7.

Extraction recoveries were determined by comparing the peak areas of extracted QC samples with peak areas of post-extraction spiked plasma blanks at corresponding concentrations. The extraction recoveries of fluconazole were determined at 2.5 and 80 ng ml^{-1} concentrations. The mean recoveries of fluconazole were 87% at 2.5 ng ml^{-1} and 91% at 80 ng ml^{-1}. The mean recovery for the IS was 93%. Acceptable and consistent recoveries were obtained for both fluconazole and IS.

Calibration curve parameters and data are listed in Table 3. The correlation coefficients of the three validation curves were all >0.996. The standards show a linear range of 0.5–100 ng ml^{-1}, using

Table 5
Stability of the samples ($n = 6$)

	Concentration (ng ml^{-1})	
	1.50	70.0
3 Freeze/thaw cycles		
Mean	1.55	72.9
RSD (%)	8.4	5.7
RE (%)	+3.2	+4.2
24 h bench-top		
Mean	1.59	72.3
RSD (%)	9.0	6.1
RE (%)	+6.1	+3.3
−20 °C for 242 days		
Mean	1.55	72.4
RSD (%)	11.7	13.2
RE (%)	+3.3	+3.4
48 h extract		
Mean	1.55	72.2
RSD (%)	7.2	2.2
RE (%)	+3.1	+3.2

Table 6
Method ruggedness

	HILIC-MS/MS system *A*, column A			HILIC-MS/MS system *B*, column B		
(ng ml^{-1})	1.50	10.0	70.0	1.50	10.0	70.0
Mean	1.48	10.1	68.5	1.56	10.4	72.9
RSD (%)	5.9	4.0	6.8	2.7	3.7	5.8
RE (%)	−1.7	+1.4	−2.2	+4.2	+4.3	+4.2

weighted (1/concentration^2) least-square linear regression.

The precision and accuracy data for QC samples are summarized in Table 4. The data show that this method is consistent and reliable with low RSDs and REs values. For the LLOQ QCs, the RSD ($n = 6$) of the measured concentration was 5.8%. The RE of the mean of the measured concentrations were −14%.

The stability tests were designed to cover the anticipated conditions that the clinical samples may experience. Stability of sample processing (freeze/thaw, bench-top and storage), and chromatography (extracts) were tested and established. The results are summarized in Table 5. Three freeze/thaw cycles and ambient temperature storage of the QC samples for up to 24 h prior to analysis, appeared to have little effect on the quantitation. QC samples stored in a freezer at −20 °C remained stable for at least 242 days. Extracted calibration standards and QC samples were allowed to stand at 2–8 °C for 48 h prior to injection. No effect on quantitation of the calibration standards or QC samples was observed. The method ruggedness was demonstrated by using multiple analytical columns and HILIC-MS/MS instruments. Acceptable results were obtained as shown in Table 6.

4. Conclusion

A sensitive, reliable and highly automated HILIC-MS/MS method for the measurement of fluconazole in human potassium EDTA plasma has been successfully developed and validated. A silica column and an aqueous–organic mobile phase were used to improve the sensitivity. The LLOQ is 0.5 ng ml^{-1} for both fluconazole by using only 0.050 ml plasma. The highly automated nature of the method significantly improved the sample analysis throughput.

References

[1] Physicians' Desk Reference®, 54th ed. Medical Economics Company, Inc., Montvale, NJ, USA, 2000, p. 2338.
[2] D. Debruyne, J.P. Ryckelynck, Clin. Pharmacokinet. 24 (1993) 10.
[3] S. Ripa, L. Ferrante, M. Prenna, Chemotherapy 39 (1993) 6.
[4] L.L. von Moltke, D.J. Greenblatt, S.X. Duan, J.S. Harmatz, R.I. Shader, Pharm. Pharmacol. Commun. 4 (1998) 443.
[5] K.E. Warren, C.M. McCully, T.J. Walsh, F.M. Balis, Antimicrob. Agents Chemother. 44 (2000) 1100.
[6] O. Lortholary, M. Nicolas, S. Soreda, L. Improvisi, O. Ronin, O. Petitjean, B. Dupont, M. Tod, F. Dromer, J. Antimicrob. Chemother. 43 (1999) 817.
[7] M.T. Borin, S.R. Cox, B.D. Herman, B.J. Carel, R.D. Anderson, W.W. Freimuth, Antimicrob. Agents Chemother. 41 (1997) 1892.
[8] G. Omar, P.H. Whiting, G.M. Hawksworth, M.J. Humphrey, M.D. Burke, Ther. Drug Monit. 19 (1997) 436.
[9] D.M. Canafax, N.M. Graves, D.M. Hilligoss, B.C. Carleton, M.J. Gardner, A.J. Matas, Transplantation 51 (1991) 1014.
[10] C.B. Trapnell, C. Jamis-Dow, R.W. Klecker, J.M. Collins, Antimicrob. Agents Chemother. 41 (1997) 924.
[11] R.A. Blum, J.H. Wilton, D.M. Hilligoss, M.J. Gardner, E.B. Henry, N.J. Harrison, J.J. Schentag, Clin. Pharmacol. Ther. 49 (1991) 420.
[12] J. Schmider, D.J. Greenblatt, L.L. von Moltke, D. Karsov, R. Vena, H.L. Friedman, R.I. Shader, J. Clin. Pharmacol. 37 (1997) 193.
[13] J.H. Rex, L.H. Hanson, M.A. Amantea, D.A. Stevens, J.E. Bennett, Antimicrob. Agents Chemother. 35 (1991) 846.
[14] R.W. Yee, C.J. Cheng, S. Meenakshi, T.M. Ludden, J.E. Wallace, M.G. Rinaldi, Cornea 16 (1997) 64.

[15] M. Cociglio, S. Brandissou, R. Alric, F. Bressolle, J. Chromatogr. B 686 (1996) 11.
[16] C.H.W. Koks, H. Rosing, P.L. Meenhorst, A. Bult, J.H. Beijnen, J. Chromatogr. B 663 (1995) 345.
[17] F.J.F. Murrieta, V.G. Soto, E. Hong, J. Liq. Chromatogr. 17 (1994) 3803.
[18] H. Zhang, R.E. Mullins, Clin. Chem. 40 (1994) 1083.
[19] T.K. Ng, R.C. Chan, F.A. Adeyemi-Doro, S.W. Cheung, A.F. Cheng, J. Antimicrob. Chemother. 37 (1996) 465.
[20] F. Von Heeren, R. Tanner, R. Theurillat, W. Thormann, J. Chromatogr. A 745 (1996) 165.
[21] K. Inagaki, J. Takagi, E. Lor, M.P. Okamoto, M.A. Gill, Ther. Drug Monit. 14 (1992) 306.
[22] J.E. Wallace, S.C. Harris, J. Gallegos, G. Foulds, C.J. Chen, M.G. Rinaldi, Antimicrob. Agents Chemother. 36 (1992) 603.
[23] K.K. Hosotsubo, H. Hosotsubo, M.K. Nishijima, T. Okada, N. Taenaka, I. Yoshiya, J. Chromatogr. 529 (1990) 223.
[24] L.A. Moraes, F.E. Lerner, M.E. Moraes, M.O. Moraes, G. Corso, G. de Nucci, Ther. Drug Monit. 21 (1999) 200.
[25] G. de Nucci, F.A.F. Bezerra, F.E. Lerner, M.E. Moraes, W. Ribeiro, E.A. Zappi, Arzneimittel-Forschung 50 (2000) 1028.
[26] J. Janiszewski, R.P. Schneider, K. Hoffmaster, M. Swyden, D. Wells, H. Fouda, Rapid Commun. Mass Spectrom. 11 (1997) 1033.
[27] L. Ramos, R. Bakhtiar, F.L.S. Tse, Rapid Commun. Mass Spectrom. 14 (2000) 740.
[28] Z. Shen, S. Wang, R. Bakhtiar, Rapid Commun. Mass Spectrom. 16 (2002) 332.
[29] W. Naidong, Y.-L. Chen, W.Z. Shou, X. Jiang, J. Pharm. Biomed. Anal. 26 (2001) 753.
[30] W. Naidong, W.Z. Shou, Y.-L. Chen, X. Jiang, J. Chromatogr. B 754 (2001) 387.
[31] W. Naidong, J.W. Lee, X. Jiang, M. Wehling, J.D. Hulse, P.P. Lin, J. Chromatogr. B 735 (1999) 255.
[32] W. Naidong, X. Jiang, K. Newland, R. Coe, P.P. Lin, J.W. Lee, J. Pharm. Biomed. Anal. 23 (2000) 697.
[33] W.Z. Shou, X. Jiang, B.D. Beato, W. Naidong, Rapid Commun. Mass Spectrom. 15 (2001) 466.
[34] W.Z. Shou, M. Pelzer, T. Addison, X. Jiang, W. Naidong, J. Pharm. Biomed. Anal. 27 (2002) 143.
[35] A. Eerkes, T. Addison, W. Naidong, J. Chromatogr. B 768 (2002) 277.
[36] Y.-L. Chen, G.D. Hanson, X. Jiang, W. Naidong, J. Chromatogr. B 769 (2002) 55.
[37] W.Z. Shou, H.-Z. Bu, T. Addison, X. Jiang, W. Naidong, J. Pharm. Biomed. Anal. 29 (2002) 83.
[38] W. Naidong, H.-Z. Bu, Y.-L. Chen, W.Z. Shou, X. Jiang, T.D.J. Halls, J. Pharm. Biomed. Anal. 28 (2002) 1115.
[39] W. Naidong, W.Z. Shou, T. Addison, S. Maleki, X. Jiang, Rapid Commun. Mass Spectrom. 16 (2002) 1965.
[40] R.D. Voyksner, in: R.B. Cole (Ed.), Electrospray Ionization Mass Spectrometry, Wiley, New York, 1997, p. 323.
[41] P.E. Kuhlmann, A. Apffel, S.M. Fischer, G. Goldberg, P.C. Goodley, J. Am. Soc. Mass Spectrom. 6 (1995) 1221.
[42] M. Jemal, D. Teitz, O. Zheng, S. Khan, J. Chromatogr. B 732 (1999) 501.
[43] B.K. Matuszewski, M.L. Constanzer, C.M. Chavez-Eng, Anal. Chem. 70 (1998) 882.
[44] I. Fu, E.J. Woolf, B.K. Matuszewski, J. Pharm. Biomed. Anal. 18 (1998) 347.
[45] D.L. Buhrman, P.I. Price, P.J. Rudewicz, J. Am. Soc. Mass Spectrom. 7 (1996) 1099.
[46] P. Kebarle, Y. Ho, in: R.B. Cole (Ed.), Electrospray Ionization Mass Spectrometry, Wiley, New York, 1997, pp. 42–50.
[47] S. Zhou, M. Larson, W. Naidong, X. Jiang, Proceeding of the 50th ASMS conference on mass spectrometry and Allied Topics, June 2-6, 2002, Orlando, Florida, USA.