J Huazhong Univ Sci Technol [Med Sci]
DOI 10.1007/s11596-014-1296-y
34(3):431-436,2014

Development of a Liquid Chromatography-tandem Mass Spectrometry Method for Determination of Butoconazole Nitrate in Human Plasma and Its Application to a Pharmacokinetic Study*

Meng-meng JIA (贾萌萌), Ying ZHOU (周 莹), Xiao-meng HE (何晓梦), Yi-lai WU (吴义来), Hu-qun LI (李虎群), Hui CHEN (谌 辉)#, Wei-yong LI (黎维勇)#
Institute of Clinical Pharmacy, Union Hospital, Tongji Medical College, Huazhong University of Science and Technology, Wuhan 430022, China

Summary: A liquid chromatography-tandem mass spectrometry (LC-MS/MS) method was developed and validated for the determination of butoconazole in human plasma. Human plasma samples of 0.2 μL were pretreated by a single step protein precipitation procedure and analyzed using a high performance liquid chromatography (HPLC) electrospray tandem mass spectrometer system. The compounds were eluted isocratically on an Inertsil ODS-SP column (100 mm×2.1 mm, 3 μm), ionized using a positive ion atmospheric pressure electrospray ionization source and analyzed using multiple reaction monitoring (MRM) mode. The ion transitions monitored were *m/z* 412.8→165.1 for butoconazole and *m/z* 453.4→230.3 for the internal standard. The chromatographic run time was 3.5 min per injection, with retention time of 2.47 min and 2.15 min for butoconazole and repaglinide, respectively. The method was validated to be linear over the range of 20 to 8000 pg/mL (r>0.999) by using a weighted ($1/x^2$) quadratic regression. The mean recovery rate was more than 86.7%, and the intra- and inter-day precision of the quality control samples (QCs) was less than 8.3% and the accuracy ranged from 96.0% to 110.2%, which indicated that the quantitative method was reliable and accurate. The method is simple, rapid, and has been applied successfully to a pharmacokinetics study of butoconazole nitrate suppositories in healthy Chinese females.
Key words: liquid chromatography-tandem mass spectrometry; human plasma; pharmacokinetics; butoconazole nitrate

Butoconazole nitrate [(±)-1-(4-Chlorophenyl)-2-(2,6-dichlorophenylthio)-n-butyl imidazole nitrate], an imidazole developed by Syntex Research[1, 2], is active as an antifungal agent *in vivo* and *in vitro*. It exists as a racemic mixture in which both components are optical isomers and equally active[2–5]. Butoconazole nitrate has been singled out for clinical studies since it is proven to be more effective than either miconazole nitrate (MN) or clotrimazole in experimental vaginal candidiasis[6–8]. The chemical structure of butoconazole nitrate is shown in fig. 1.

Up to now, few methods were reported to determine butoconazole in plasma. An high performance liquid chromatography (HPLC) method was described for the determination of butaconazole[9]. Senchenko *et al* reported a capillary electrophoresis method applied to the determination of butoconazole in rat blood after intraperitoneal administration[10]. However, the method is not sensitive enough for detecting butoconazole in the human plasma after vaginal administration of butoconazole nitrate suppositories, and the reported method requires analysis time as long as 18 min. Therefore, a simple, sensitive and rapid liquid chromatography-tandem mass spectrometry (LC-MS/MS) method was developed for the determination of butoconazole in human plasma with the limit of quantification of 20 pg/mL, and has also been successfully applied to evaluate the pharmacokinetics of butoconazole nitrate after vaginal administration of 100 mg suppositories of butoconazole nitrate to 8 healthy Chinese females.

1 MATERIALS AND METHODS

1.1 Chemicals and Reagents

Butoconazole nitrate suppositories and butoconazole nitrate reference standard (99.6% purity) were supplied by Xi'an Lijun Pharmaceutical Co., Ltd. (China). Repaglinide reference standard (internal standard, IS, 98.6% purity) was purchased from National Institute for the Control of Pharmaceutical and Biological Products

Meng-meng Jia, E-mail: wjsjiamengmeng@163.com
#Corresponding authors, Wei-yong LI, E-mail: 2621239868@qq.com; Hui CHEN, E-mail: 15902710359@163.com
*This project was supported by National Science & Technology Specific Projects in the 12th Five-Year Plan of China (No. 2011ZX09302-002-01).

(China). HPLC grade methanol, acetonitrile and ammonium formate were all purchased from Dikma Technologies Co. Ltd. (China). HPLC-grade formic acid was purchased from the Tedia Company Inc. (Tedia, USA). Purified water used throughout the study was commercially available (Wahaha® Hangzhou Wahaha Co., Ltd, China). Fresh frozen healthy human plasma was collected from Union Hospital, Tongji Medical College, Huazhong University of Science and Technology (HUST, China). Other chemicals and reagents were of analytical grade and obtained commercially.

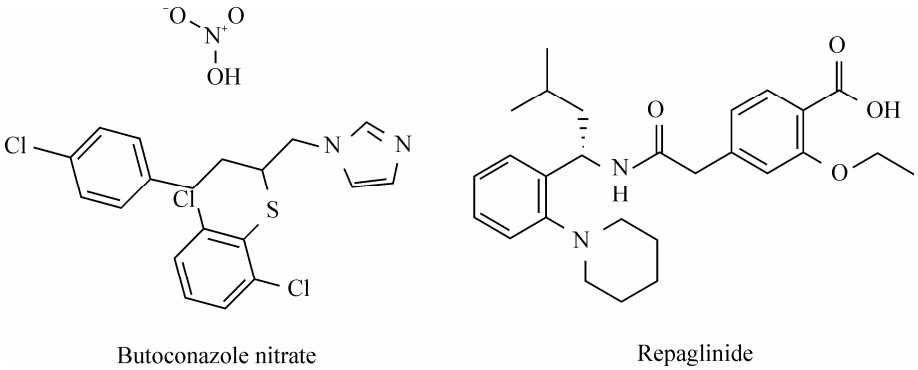

Fig. 1 Structures of butoconazole nitrate and repaglinide (IS)

1.2 Instrumentation and LC-MS/MS Conditions

An Agilent 1200 high performance liquid chromatography system equipped with quaternary pump, degasser, autosampler and column oven was used in this study. The column was Inertsil ODS-SP (2.1 mm×100 mm, 3 μm, GL Sciences Inc., Japan) and was operated at 35°C. The mobile phase consisted of acetonitrile: 20 mmol/L ammonium acetate containing 0.1% formic acid (78:22, v/v) which was set at an isocratic flow rate of 0.30 mL/min, and the injection volume was 5 μL and the run time was 3.5 min. The autosampler was kept at 4°C. Under these conditions, the retention time for butoconazole and the IS were 2.47 min and 2.15 min, respectively.

Mass spectrometric detection was performed using an API 4000 triple quadrupole instrument (AB/MDSSciex, Canada). Electrospray ionization mass spectrometry (ESI-MS) was performed, and positive ion was detected in multiple reaction monitoring (MRM) mode. All analytes were assayed by quantifying the $[M+H]^+$ ions with butoconazole detected at *m/z* 412.8 and IS at 453.4. The product ion chromatograms are shown in fig. 2, and the specific parameters for each analyte are shown in table 1. MRM data were acquired and the chromatograms were integrated with the software version: Analyst 1.6.1 software (Applied Biosystems, USA).

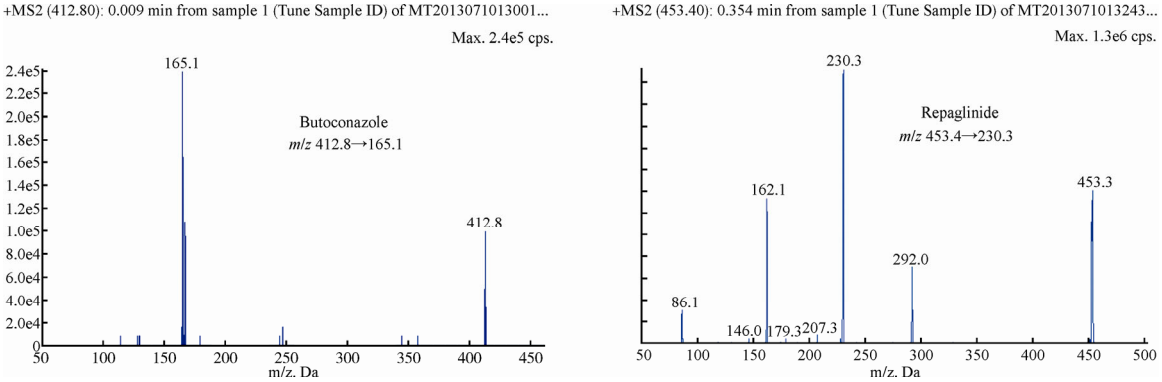

Fig. 2 MS-MS spectra of butoconazole and repaglinide (IS)

Table 1 Optimized mass parameters for butoconazole and the internal standard

Analytes	MRM (*m/z*)	Dwell time (ms)	DP (V)	CE (V)	CXP (V)
Butoconazole	412.8/165.1	200	65	25	13
Repaglinide	453.4/230.3	200	81	39	16

DP: declustering potential; CE: collision energy; CXP: collision cell exit potential

1.3 Preparation of Calibration Standards and Quality Control (QC) Samples

Standard stock solutions of butoconazole nitrate and the IS (repaglinide) were individually prepared in methanol at 583.2 and 495.5 μg/mL, respectively. Working solutions of butoconazole were prepared daily with methanol by appropriate dilution at 200, 500, 1000, 3000, 10 000, 30 000 and 80 000 pg/mL. The internal standard working solution was diluted to 200 ng/mL with methanol. Calibration samples were freshly prepared by spiking appropriate amount of the working solutions of butoconazole nitrate into 180 μL blank plasma to obtain the concentration in the range of 20–8000 pg/mL. QC solutions and QC plasma samples (60, 600 and 6400 pg/mL) were prepared in the same way. All solutions described above were stored at 4°C.

1.4 Sample Preparation

In a 2 mL Eppendorf tube containing 200 μL plasma sample, 20 μL of IS working solution (200 ng/mL) and 1 mL acetonitrile were added to precipitate plasma proteins. The mixture was vortex-mixed thoroughly for 2 min and then centrifuged at 14 000 r/min for 10 min at 4°C. Then an aliquot of 5 μL supernatant solution was injected into the LC-MS/MS system.

1.5 Method Validation

The method was validated for selectivity, sensitivity (lower limit of quantification, LLOQ), linearity, accuracy and intra-day and inter-day precision, recovery, matrix effect and stability according to the Food and Drug Administration (FDA) guideline[11] for validation of bioanalytical method. The selectivity was investigated by preparing and analyzing six individual human blank plasma samples without the presence of internal standards. Linearity was evaluated by plotting the peak area ratio (y) of the analyte to IS *versus* analyte concentration (x) in the freshly prepared plasma calibrators. Calibration curves were analyzed by weighted linear regression ($1/x^2$) of the peak area ratio of the analyte to the IS *versus* nominal concentrations. LLOQ was defined as the lowest concentration on the calibration curve at which precision was within 20% and the accuracy was within ±20%. It was tested with five samples independent of standards.

The precision and accuracy of the assay were determined by analyzing QC samples at three different validation batches on three consecutive validation days, and each batch contained a set of calibration standards and five replicates of QC samples at three different concentrations (60, 600, 6400 pg/mL). The concentrations were calculated based on calibration curve. The precision was defined as the relative standard deviation (RSD, %) and the accuracy was expressed as the relative error (RE) between the mean measured concentration and the nominal concentration. The acceptance criteria for intra-day and inter-day precision were within ±15% and accuracy did not exceed 15%.

The recovery rate of butoconazole from human plasma was determined by comparing the peak areas of the QC samples with those from the same amount of butoconazole added with the extract from the blank plasma samples. The matrix effect of butoconazole was evaluated by comparing the peak areas of butoconazole added into the extract of the blank plasma with the peak areas of the same amount of butoconazole added into the mobile phase. Experiments were performed in triplicate at three different levels, 60, 600 and 6400 pg/mL. The recovery rate and matrix effect of IS were determined in a similar way at 200 ng/mL.

Stability of analyte was examined in stock solutions, working solution and plasma samples by determining three concentrations (60, 600 and 6400 pg/mL) in triplicate under different conditions. The long-term stability was checked after storage of the plasma samples at –70°C for 28 days. The short-term stability was assessed after the exposure of the spiked samples at room temperature for 8 h. The post-preparative stability was measured after exposure of processed samples at 4°C for 6 h. The freeze-thaw stability was checked after three cycles. The stock solution stability was also assessed.

1.6 Applications in Pharmacokinetic Studies

The LC-MS method developed was used to investigate the plasma profile of butoconazole after single dose of 100-mg suppositories of butoconazole nitrate. The protocol of this study was approved by Ethics Committee of Union Hospital, Tongji Medical College, HUST (China). Eight healthy female Chinese volunteers, aged 20–45 years, participated in the study. After signing informed consent, the volunteers were vaginally administrated a single dose of butoconazole nitrate suppositories 100 mg following an overnight fast. Blood samples were collected at pre-dose (0 h) and at 1, 3, 6, 8, 10, 12, 15, 24, 36, 48, 72, 96 h after administration. Samples were separated by centrifugation immediately at 3500 r/min×10 min at 4°C and stored at –70°C until analysis. Before analysis, the plasma samples were thawed at 4°C.

2 RESULTS

The specificity was examined by comparing the chromatograms of six different batches of blank human plasma with those of the corresponding analytes in spiked plasma. As shown in fig. 3, butoconazole and repaglinide (IS) were well separated, with the retention time of 2.47 min and 2.15 min, respectively. No interference and a low background noise were observed.

The calibration curves of the analyte showed excellent linearity over the concentration range from 20 to 8000 pg/mL for butoconazole. The regression parameters of slope, intercept and correlation coefficient were calculated by $1/x^2$ weighted linear regression in Analyst 1.6.1 software. The regression equation for calibration curves were $y=(4.82\pm0.25)\times10^{-5}x+(1.24\pm0.49)\times10^{-3}$ (r=0.9986±0.0011), where y represents the peak area ratio (y) of the analyte to IS, and x represents the concentration of the analyte. The current assay offered a LLOQ of 20 pg/mL, which is sensitive enough to investigate the pharmacokinetic behaviors of butoconazole nitrate suppositories. Typical LC-MS/MS chromatogram of the LLOQ sample is shown in fig. 3.

The details of the intra- and inter-day precision and accuracy for the analytes are shown in table 2. The results were within the acceptable limits to meet the guideline for bioanalytical methods[12], which indicated the method was precise and accurate.

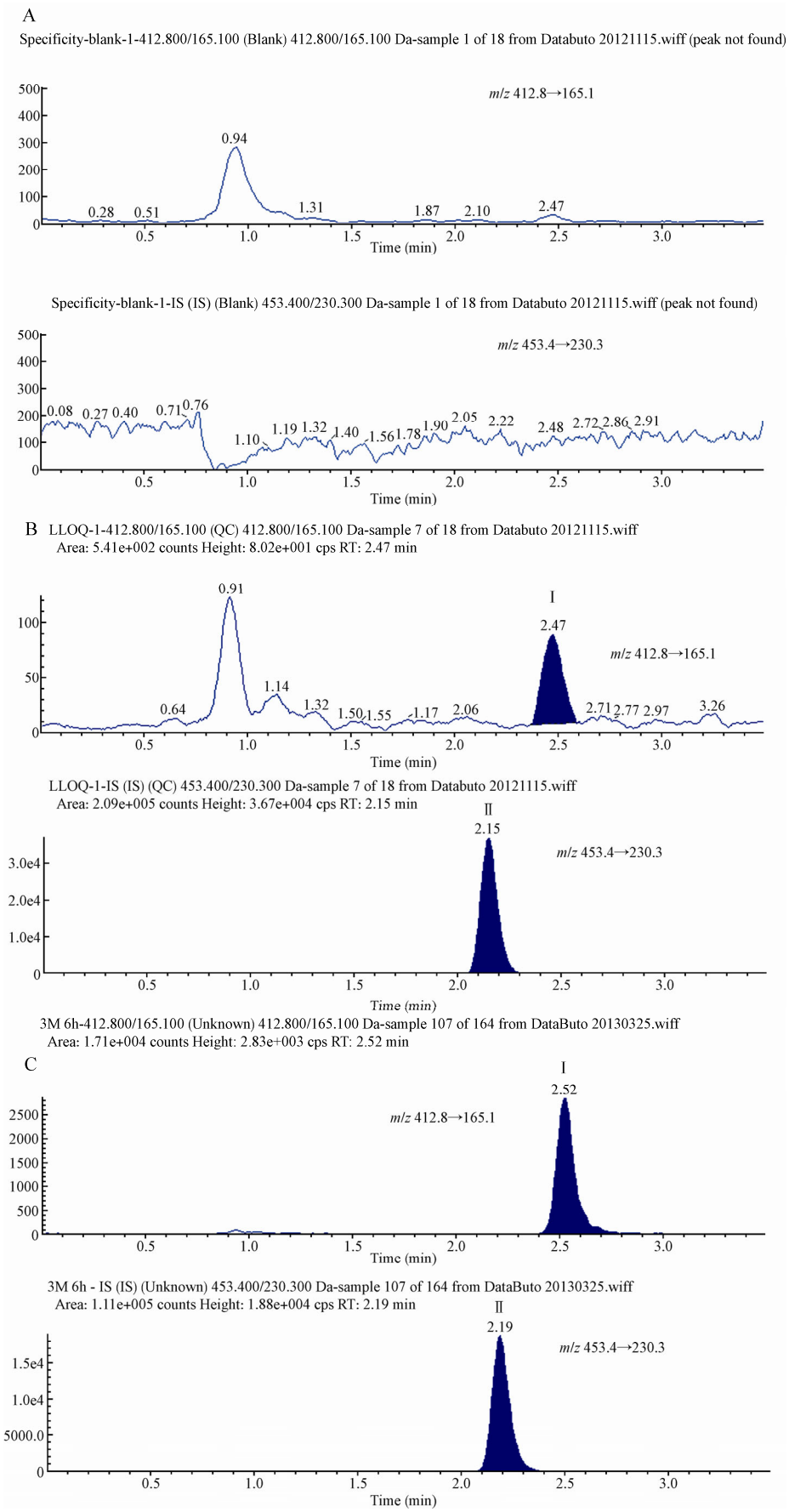

Fig. 3 Representative MRM chromatograms for butoconazole (peak I), and repaglinide (peak II, IS) from (A) blank plasma; (B) LLOQ, 20 pg/mL for butoconazole; (C) plasma sample, 6 h after administration

The extraction recovery rates of butoconazole from human plasma were (87.4±7.2)%, (88.4±7.6)%, and (86.7±4.3)% at concentration levels of 60, 600 and 6400 pg/mL, respectively, and the mean extraction recovery rate of repaglinide was (90.4±6.6)%. The matrix effects were (78.4±4.2)%, (81.4±5.6)%, and (80.7±4.9)% for butoconazole, at low, medium and high QC levels, respectively (n=5), and (86.7±3.3)% for repaglinide. No significant matrix effect for butoconazole and the IS was observed, indicating that no endogenous substance significantly influenced the ionization of the analytes.

The stock solution of butoconazole nitrate in plasma was found to be stable at room temperature for 8 h, at 4°C for 6 h, at −70°C for 28 days, at freeze and thaw stability (table 3). The stock solutions were stable for at least 3 weeks. The difference between the fresh samples and the test solution in stock solution stability was <5% for butoconazole nitrate and repaglinide, respectively. The results from all stability tests presented a good stability of butoconazole over all steps of the determination.

Table 2 Intra-day and inter-day precision and accuracy for assay of butoconazole in human plasma (on 3 consecutive days, 5 replicates for each day)

Analytes	Analyte concentration (pg/mL)	Intra-day (n=15)			Inter-day (n=15)		
		Calculated concentration ($\bar{x}$±s, pg/mL)	Precision (%)	Accuracy (%)	Calculated concentration ($\bar{x}$±s, pg/mL)	Precision (%)	Accuracy (%)
Butoconazole	60	61.4±3.2	5.2	102.3	57.6±3.5	6.1	96.0
	600	617.8±38.3	6.2	103.0	582.7±38.2	6.5	97.1
	6400	6350.0±488.8	7.6	99.2	6613.3±547.5	8.3	110.2

Table 3 Stability of butoconazole in human plasma at three concentraion levels (n=5)

Stability conditions	Accuracy (mean±RSD) (%)		
	60 (pg/mL)	600 (pg/mL)	6400 (pg/mL)
Short-term stability	96.4±5.2	101.4±5.2	95.3±3.2
Freeze-thaw stability	95.5±7.3	99.8±8.3	102.6±5.3
Long-term stability	103.8±7.4	102.4±7.3	98.8±4.3
Post-preparative stability	98.3±8.8	100.0±6.8	101.1±5.8

Results are expressed as RE (%).

The validated method was successfully used to determine plasma concentrations of butoconazole after single intravaginal administration of 100 mg suppositories of butoconazole nitrate to 8 healthy Chinese females. Mean plasma concentration-time curve of butoconazole is shown in fig. 4. Pharmacokinetic parameters were calculated using DAS 3.0 software. Analysis of butoconazole concentrations in plasma samples from 8 healthy Chinese females following intravaginal administration of 100 mg of butoconazole nitrate suppositories provided the following pharmacokinetic parameters ($\bar{x}$±s): the C_{max} and T_{max} were 4471.25±1795.991 pg/mL and 11.125±5.743 h, respectively; plasma concentration declined with the $t_{1/2}$ of 17.834±3.695 h; the $AUC_{0\text{-}t}$ and $AUC_{0\text{-}\infty}$ values obtained were 110744.756±49366.446 and 113290.135±50417.045 pg·h·mL^{-1}, respectively. The observed values of the pharmacokinetic parameters were comparable to those reported for butoconazole nitrate in previous studies[2].

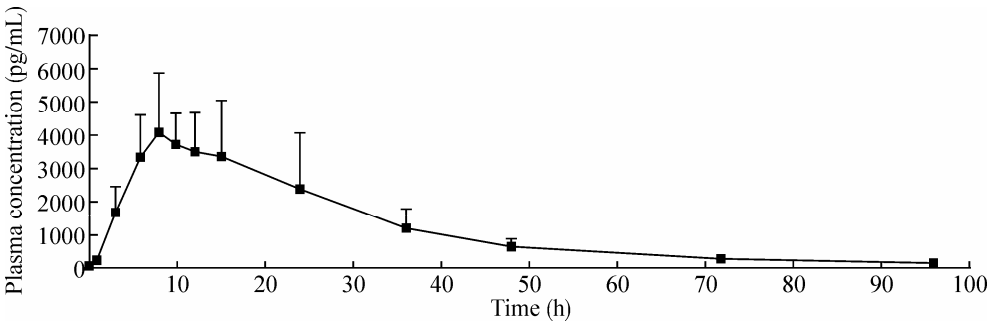

Fig. 4 Mean plasma concentration time profile of butoconazole after intravaginal administration of 100 mg of suppositories of butoconazole nitrate

3 DISCUSSION

The chromatographic separation was achieved on an Inertsil ODS-SP column (2.1 mm×100 mm, 3.0 μm, GL Sciences Inc. Japan) at temperature of 35°C. The mobile phase consisted of acetonitrile: 20 mmol/L ammonium formate containing 0.1% formic acid (78:22, v/v) at an isocratic flow rate of 0.30 mL/min.

The chromatographic conditions described in this assay were reached after investigating several mobile phases and solid phase extraction supports. Various combinations of methanol, acetonitrile, formic acid and acetic acid were investigated to optimize the mobile phase for sensitivity, retention time and peak shape. The inclusion of 20 mmol/L ammonium acetate instead of pure water reduced matrix effects without decreasing response. Peak shape was improved by using 0.1% formic acid.

After a number of C18 columns were evaluated, an Inertsil ODS-SP column gave the better chromatogram with the total LC run time of 3.5 min using isocratic elution at a flow rate of 0.30 mL/min. Under optimized HPLC conditions, the suitable retention time of butoconazole and IS was 2.47 min and 2.15 min, respectively.

Repaglinide was adopted as the appropriate IS. Although repaglinide and butoconazole are not similar in their structure, they had not only the similar chromatographic and mass spectrometric behavior, but also the similar matrix effect and extract recovery. So we chose repaglinide as IS.

In conclusion, an LC-MS/MS method for quantitation of butoconazole in human plasma has been successfully developed and validated. The method is simple, rapid, and sensitive. The lower limit of quantitation was 20 pg/mL, and the entire detecting time is less than 3.5 min. This method has been successfully applied to a pharmacokinetic study of butoconazole nitrate in healthy Chinese females.

Conflict of Interest Statement

The authors declare that there is no conflict of interest with any financial organization or corporation or individual that can inappropriately influence this work.

REFERENCES

1 Walker KA, Braemer AC, Hitt S, *et al*. 1-[4-(4-Chlorophenyl)-2-(2,6-dichlorophenylthio)-n-butyl]-1H-imidazole nitrate, a new potent antifungal agent. J Med Chem, 1978,21(8):840-843

2 Tatum DM, Eggleston M. Butoconazole nitrate. Infect Cont Hosp Epidemiol, 1988, 9(3):122-124

3 Droegemueller W, Adamson DG, Brown D, *et al*. Three-day treatment with butoconazole nitrate for vulvovaginal candidiasis. Obstet Gynecol, 1984,64(4): 530-534

4 Jacobson JB, Hajman AJ, Wiese J, *et al*. A new vaginal antifungal agent-butoconazole nitrate. Acta Obstet Gyn Scan, 1985,64(3):241-244

5 Bradbeer CS, Mayhew SR, Barlow D. Butoconazole and miconazole in treating vaginal candidiasis. Genitourin Med, 1985,61(4):270-272

6 Brown Jr D, Henzl MR, LePage ME, *et al*. Butoconazole vaginal cream in the treatment of vulvovaginal candidiasis. Comparison with miconazole nitrate and placebo. J Reprod Med, 1986,31(11):1045

7 Seidman LS, Skokos CK. An evaluation of butoconazole nitrate 2% Site Release® vaginal cream (Gynazole-1®) compared to fluconazole 150 mg tablets (Diflucan®) in the time to relief of symptoms in patients with vulvovaginal candidiasis. Infect Dis Obstet Gynecol, 2005,13(4):197-206

8 Hajman AJ. Vulvovaginal candidosis: comparison of 3-day treatment with 2% butoconazole nitrate cream and 6-day treatment with 1% clotrimazole cream. J Int Med Res, 1988,16(5):367

9 The United States Pharmacopeial Convention XXIV. US Pharmacopeia/National Formulary (USP35-NF30). Washington: US Pharmacopeia Origination Press, 2013

10 Senchenko SP, Checheneva KS, Gavrilin MV, *et al*. Butoconazole nitrate pharmacokinetics studied by capillary electrophoresis. Pharm Chem J, 2009,43(11):597-600

11 Food and Drug Administration, Center for Drug Evaluation and Research (CDER) Guidance for Industry, Bioanalytical Method Validation, US Department of Health and Human Services, May 2001

12 U.S. FDA, Rockville, MD: CDER (2001)

(Received Nov. 8, 2013; revised Feb. 21, 2014)