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Solid phase extraction procedure coupled with the chiral LC-ESI-MS/MS method for the enantioseparation and determination of butoconazole enantiomers in rat plasma and tissues: application to the enantioselective study on pharmacokinetics and tissue distribution†

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In the present study, a highly rapid, sensitive and enantioselective method was developed and fully validated for the separation and determination of butoconazole enantiomers in rat plasma and tissues by liquid chromatography–electrospray ionization coupled with tandem mass spectrometry (LC-ESI-MS/MS). The analytes and internal standard (tioconazole) were both extracted from plasma and tissue samples by the solid phase extraction (SPE) procedure with C_{18} cartridges. Satisfactory enantioseparation was achieved on a Chiralpak IC column by using acetonitrile/10 mM aqueous ammonium acetate (90 : 10, v/v) as a mobile phase. Butoconazole enantiomers and IS were detected in the multiple reaction monitoring (MRM) mode with a positive electrospray ionization source. A comprehensive validation of this method was conducted over the concentration range of 0.5–250 ng mL^{-1}, and good linearity was obtained for each enantiomer with correlation coefficient (R^2) greater than 0.991. The mean extraction recoveries were higher than 90.4%, and the relative error was well within the admissible range of −8.0 to 9.1% and the relative standard deviation was less than 11.5%. All the validation data demonstrated that the desirable specificity, carry-over, linearity, sensitivity, accuracy, precision, extraction recovery, matrix effect and stability were attained from the proposed approach. After validation, the established method was successfully applied to the study on stereoselective pharmacokinetics and tissue distribution in female Sprague-Dawley rats after transdermal administration of 10 mg kg^{-1} 2% *rac*-butoconazole nitrate cream. It should be noted that this is the first report regarding the stereospecific study of butoconazole enantiomers *in vivo*.

1. Introduction

More than half of clinical drugs are chiral compounds with one or more chiral centers. After *in vivo* adminstration, in most cases, one enantiomer is pharmacologically active but the other may be inactive or could exert toxic effects. Thus, quantitative assay of individual enantiomers of chiral drugs in biological fluids and tissues is an extraordinarily pivotal step to highlight the stereoselective implication, therapeutic application, and toxicology of chiral drugs.[1,2]

Butoconazole, as an antifungal prescription medicine approved by the US Food and Drug Administration (US FDA), is widely used for the topical treatment of vaginal candidiasis.[3] Butoconazole contains one asymmetric carbon and possesses two enantiomers (Fig. 1), but is still clinically used as racemates.[4] Although several different methods have been used to determine the *rac*-butoconazole concentrations in plasma, including liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS)[3,4] and capillary electrophoresis (CE),[5] to date, no information is available on the pharmacokinetic and pharmacodynamic properties of individual enantiomers of butoconazole. Besides, for the two enantiomers of the chiral azole antifungal, some previous papers revealed that the obvious enantioselective behavior between the two azole enantiomers was observed not only during metabolism, but also during the

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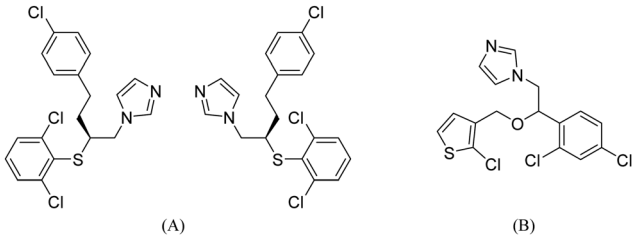

Fig. 1 Chemical structure of butoconazole (A) and IS (B).

various stages of ADME (absorption, distribution, metabolism, and excretion). According to the study by Du *et al.* the concentration of *R*-(−)-miconazole in rat plasma was approximately 1.8 times higher than that of antipode after administration of *rac*-miconazole.[6] Meanwhile, Hamdy *et al.* confirmed that the concentration of (+)-ketoconazole in plasma was approximately two-fold higher than that of (−)-ketoconazole.[7] In assays for fenticonazole enantiomers, *S*-(+)-fenticonazole exhibited faster absorption and elimination in female rats compared to *R*-(−)-fenticonazole.[8] Therefore, it is very urgent and important to explore the potential stereoselectivity of butoconazole enantiomers *in vivo*.

Chiral high performance liquid chromatography (HPLC) using the chiral stationary phase (CSP) method was proved to be the most effective and convenient technique for chiral separation. Zhu *et al.*[9] investigated the enantioseparation abilities of four immobilized polysaccharide-derived CSPs (Chiralpak IA, Chiralpak IB, Chiralpak IC and Chiralpak ID) in the normal phase mode, and found that all these four chiral columns showed excellent enantioselectivity toward *rac*-butoconazole. Recently, butoconazole enantiomers were resolved in the normal phase mode using a derivatized 4-chlorophenylcarbamate-β-cyclodextrin bonded chiral stationary phase with *n*-hexane and isopropanol as a mobile phase by our group.[10] In these studies, the separation of butoconazole enantiomers was performed under the normal phase mobile phase conditions. However, the reversed phase separation mode was much more preferred and recommended for *in vivo* analysis due to its better compatibility for HPLC with MS detection and solubility for biological matrices.[11] Therefore, it is necessary to develop a convenient reversed phase method for the separation of butoconazole enantiomers.

Considering the obvious hepatotoxicity, butoconazole is mainly administered transdermally or intravaginally for clinical usage, resulting in a low blood and tissue concentration. Thus, a novel and sensitive chiral LC-ESI-MS/MS method was developed and applied for the determination of butoconazole enantiomers in rat plasma and tissues (kidneys, liver, heart, lungs and brain) to study the enantioselective pharmacokinetics and tissue distribution. The extraction of butoconazole from rat plasma and tissues was performed with an efficient solid phase extraction (SPE) method. The Chiralpak IC column was selected to separate the butoconazole enantiomers in the reversed phase mode and under chromatographic conditions, including the thoroughly optimized type and proportion of buffer solutions and organic modifiers. The proposed method was fully validated in compliance with the FDA guidelines and successfully applied to the study of enantioselective pharmacokinetics and tissue distribution in female Sprague-Dawley rats following transdermal administration of 10 mg kg^{-1} 2% *rac*-butoconazole nitrate cream. Accordingly, the results obtained in the present work provided, for the first time, experimental evidence of the enantioselective behavior of butoconazole *in vivo*.

2. Experimental

2.1 Chemicals and reagents

2% *rac*-butoconazole nitrate cream was purchased from the General Hospital of Shenyang Military Region (Shenyang, China). *rac*-Butoconazole (98.0% purity) and tioconazole (Internal Standard, 99.0% purity) were obtained from the National Institute for Food and Drug Control (Beijing, China). MS-grade ammonium acetate, acetic acid, methanol (MeOH) and acetonitrile (ACN) were purchased from Sigma Aldrich (Beijing, China). Ultrapure water was purified with a Milli-Q water system (Millipore, MA, USA).

2.2 Preparation of standard and quality control solutions

The stock solutions of *rac*-butoconazole and IS were prepared independently in acetonitrile at a concentration of 1.0 mg mL^{-1}. The stock solution of *rac*-butoconazole was further diluted in acetonitrile to obtain a series of working standard solutions ranging from 5.0 to 2500 ng mL^{-1} for each enantiomer. The IS working solution was prepared by diluting the IS stock solution in acetonitrile to the desired concentration. The calibration standards of butoconazole in rat plasma and tissues were prepared by adding 20 μL of standard working solutions into 200 μL of the blank plasma and the tissue homogenate. Then, the final plasma concentrations for each enantiomer were 0.5–50 ng mL^{-1} (1.0–250 ng mL^{-1} for liver and kidneys; 1.0–100 ng mL^{-1} for heart; 2.0–200 ng mL^{-1} for lungs). All quality control (QC) samples were prepared at 2.0, 20, and 40 ng mL^{-1} for each butoconazole enantiomer in rat plasma by the same steps (2.5, 50, 200 ng mL^{-1} for liver and kidneys; 2.5, 40, 80 ng mL^{-1} for heart; 8.0, 50, 160 ng mL^{-1} for lungs). Then, all the solutions were stored at 4 °C.

2.3 Sample preparation

2.3.1 Pretreatment of plasma samples. Butoconazole enantiomers were extracted from plasma samples using a solid phase extraction (SPE) method with the Cleanert C_{18} cartridge (Agela, 500 mg, 3 mL). Briefly, 200 μL plasma sample was transferred into a 1.5 mL polypropylene microcentrifuge tube, followed by the addition of 20 μL IS working solution. The tube was vortexed for 1 min. The cartridge was preconditioned with 3 mL of methanol and then equilibrated with 3 mL of pure water. Then, the sample solution was loaded onto the SPE column. When the sample had eluted under gravity, the column was washed with pure water (3 mL). Vacuum was applied to the cartridge for 3 min to completely dry the resin. Finally, elution was carried out with

3 mL of acetonitrile into the pre-labeled tubes, and the eluate was evaporated to dryness under a gentle stream of nitrogen at room temperature. The obtained residue was reconstituted with 200 μL mobile phase and vortexed for 1 min. After filtering through a 0.22 μm nylon syringe filter, an aliquot of 10 μL was injected into the analytical system for LC-ESI-MS/MS analysis.

2.3.2 Pretreatment of tissue samples. The rats after execution from each group were quickly separated on an ice bag and divided into different tissues (kidneys, liver, heart, lungs and brain). After washing three times with 0.9% sodium chloride solution, the tissues were weighed precisely (about 0.1 g) and homogenized in an ice bath with a 10-fold (w/v) volume of water containing 5% methanol. Afterward, 200 μL of the tissue homogenate was transferred into a 1.5 mL polypropylene microcentrifuge tube for the SPE procedure in the same way as the pretreatment of the plasma samples in Section 2.3.1.

2.4 Chiral LC-MS/MS conditions

Chromatographic analysis was performed on an Acquity™ UPLC system (Waters Corp. Milford, MA, USA) equipped with an Acquity™ UPLC binary solvent management system, an Acquity™ UPLC autosampler and a thermostatic column compartment. Chiral separation of the butoconazole enantiomers was achieved on a Chiralpak IC column (250 mm × 4.6 mm, 5 μm) protected with a Chiralpak IC guard column (10 mm × 4 mm, 5 μm) (Daicel, Japan). The mobile phase was acetonitrile/10 mM aqueous ammonium acetate (90 : 10, v/v) with a flow rate of 0.6 mL min^{-1}. The column and autosampler temperatures were set at 25 and 4 °C, respectively.

A triple quadrupole mass spectrometer (Waters, Milford, USA) coupled with an electrospray ionization (ESI) source was used for the detection of butoconazole enantiomers and IS in the positive ionization mode. For two MRM transitions, the most sensitive transition was used for quantification, while the other transition was used for confirmation. The optimal MS parameters were set as follows: capillary voltage of 3.0 kV, source temperature of 500 °C, desolvation temperature of 150 °C, desolvation gas (nitrogen) flow of 1000 L h^{-1}, collision gas (argon) flow of 0.12 mL min^{-1}, and nebulizer gas pressure of 7.0 bar. For butoconazole, the cone voltage (CV) was set at 25 V and the ion pair transitions of m/z 413.15 > 165.01 and m/z 413.15 > 128.47 were used for quantification and identification, respectively. The corresponding collision energy (CE) was 12 and 30 eV, respectively. For IS, the cone voltage (CV) was set at 20 V and the ion pair transitions of m/z 388.26 > 133.11 and m/z 388.26 > 89.23 were used for quantification and identification, respectively. The corresponding CE were 10 and 16 eV, respectively.

2.5 Elution order of the enantiomers

Commercially enantiopure standards for butoconazole were not available, and the elution order on the Chiralpak IC column had not been reported. In our experiment, initially, we processed the separation of butoconazole enantiomers to obtain the enantiopure eluent on a Chiralpak IC column by using acetonitrile/10 mM aqueous ammonium acetate (90 : 10, v/v) as the mobile phase on HPLC-UV equipment. The collected eluent was evaporated to dryness under a gentle stream of nitrogen at room temperature. Then, the residue was reconstituted with 500 μL methanol. After filtering through a 0.22 μm nylon syringe filter, a volume of 400 μL was injected into an Electronic Circular Dichroism (ECD) analytical system (Bio-Logic MOS-450, Franch) for the confirmation of the configuration. The results presented that the first eluted enantiomer was (−)-butoconazole, while the second one was (+)-butoconazole in this study. The spectra are shown in Fig. 2.

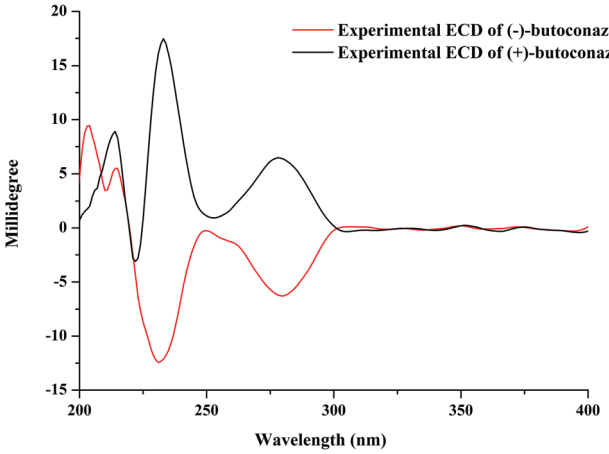

Fig. 2 Circular dichroism spectrum of *rac*-butoconazole.

2.6 Method validation

The optimized LC-MS/MS method was validated in accordance with the US Food and Drug Administration (FDA) guidelines[12] in the aspects of specificity and carry-over, the lower limit of quantification (LLOQ), linearity, accuracy and precision, matrix effect, extraction recovery and stability. Each of the QC samples were replicated 6 times and quantified by the regression equation.

2.6.1 Specificity and carry-over. The assay selectivity was determined by analyzing the extracts of multiple samples (n = 6) of plasma and tissues from different batches to evaluate the possibility of endogenous interferences around the retention time of analytes and IS. The response values of endogenous interference components co-eluted at the retention time of analytes and IS should be lower than 20% of LLOQ and 5% of IS, respectively. In terms of the carry-over, the measurement was carried out by injecting the blank plasma and tissue extracts after injecting the spiked samples at the upper limit of the quantification level. The carry-over should be lower than 20% for the LLOQ peak area and 5% for the IS peak area.

2.6.2 Linearity and LLOQ. The linearity of the developed method was determined by fitting two calibration curves containing seven nonzero levels on three consecutive days. The calibration curves ($y = ax + b$) in plasma and tissues were generated by plotting the peak-area ratios of each enantiomer to IS (y) *versus* the nominal enantiomer concentration (ng mL^{-1}, x) in the spiked samples by the $1/x^2$ weighted least squares linear regression.

The acceptable criterion was that the correlation coefficient (R^2) must be more than 0.99. The LLOQ value was defined as the minimum concentration of each enantiomer in the plasma and tissue samples that can be reliably quantified with a signal-to-noise ratio (S/N) $\geq$ 10. The accuracy of each spiked LLOQ sample (n = 6) was limited within $\pm$20%, and the precision should be not more than 20%.

2.6.3 Accuracy and precision. The accuracy and precision of the proposed method were evaluated by analyzing the QC samples in six replicates within one day (intra-day) and during three consecutive days (inter-day). The intra- and inter-day precisions were expressed as the relative standard deviation (RSD), while the relative error (RE) was employed to evaluate accuracy. For successful method validation, the intra- and inter-day precision values were required not to exceed 15% and the accuracy was required to be within $\pm$15%.

2.6.4 Extraction recovery and the matrix effect. The extraction recoveries of the butoconazole enantiomers and IS in plasma and tissue samples were evaluated at the QC concentrations in six replicates. For the extraction recovery, the peak areas of each butoconazole enantiomer and IS obtained from the QC samples (spiked before extraction) were compared with those obtained from the unextracted samples (spiked after extraction). In terms of the matrix effect, the individual peak responses of each butoconazole enantiomer and IS from the unspiked samples (spiked after extraction) were compared with those prepared in neat standard solutions at an equivalent concentration (six replicates each). The IS-normalized matrix factor (NMF) was counted by using the equation as follows: NMF (%) = $MF_{analyte}/MF_{IS} \times 100\%$ and RSD of the IS-normalized matrix factor (NMF) should be less than 15%.

2.6.5 Stability. The stability of the butoconazole enantiomers in rat plasma and tissues was estimated by analyzing the *rac*-butoconazole QC samples at three QC concentration levels in six replicates. The different storage and handling conditions were as follows: (a) short-term stability at room temperature for 12 h, (b) freeze–thaw stability after three freeze–thaw cycles from -80 °C to room temperature, (c) long-term stability at -80 °C for 30 days, and (d) post-preparative stability at 4 °C for 24 h. The analytes were considered to be stable when the accuracy biases were within $\pm$15% and the RSD was lower than 15%.

2.7 Study on enantioselective pharmacokinetics and tissue distribution

Thirty female Sprague-Dawley rats with a weight of 200–220 g were kindly supplied by the Experimental Animal Centre of Shenyang Pharmaceutical University (Wenhua Road, Shenyang, PR China). The animals were randomly divided and housed in five groups (6 animals in each group) under standardized environmental conditions (22 $\pm$ 2 °C, 12 h light/dark cycle with the light on at 8 : 00 am). All rats were fasted for 18 h, but with free access to water before the experiment. The animals of group one were transdermally administered a dose of 10 mg kg^{-1} 2% *rac*-butoconazole nitrate cream. 300 μL of the orbital blood was collected into a 1.5 mL heparinized disposable polypropylene tube at pre-dose (0 h), 0.25, 0.5, 1, 2, 4, 6, 8, 10, 12, 24 and 48 h. The obtained blood samples were centrifuged at 5000 rpm for 10 min, and, then, the supernatant was collected and stored at -80 °C until analysis. The remaining groups were also transdermally administered a dose of 10 mg kg^{-1} 2% *rac*-butoconazole nitrate cream. Then, the cervical dislocation was executed after 4, 10, 24, and 48 h, and the kidneys, liver, heart, lungs and brain were rapidly separated in an ice bag. The blank tissues were obtained from the untreated animals. This study was approved by the Institute Ethical Committee for experimental Use of Animals in Shenyang Pharmaceutical University (permit number, SYPU-IACUC-C2019-6-16-202). All procedures were adhered to principles expressed in the declaration of Helsinki.

The plasma and tissue concentrations of the butoconazole enantiomers at different time points were calculated from the calibration curves. Pharmacokinetic parameters including the maximum plasma concentration (C_{max}), the time to reach the maximum plasma concentration (T_{max}), the half-life of elimination ($t_{1/2}$), the area under the plasma concentration curve from 0 to 48 h (AUC_{0-48}), the area under the plasma concentration curve from 0 to infinity ($AUC_{0-\infty}$), mean retention time from 0 to 48 h (MRT_{0-48}) and apparent clearance (CLz/F) were obtained from DAS 2.0 software (Chinese Pharmacological Society) using the non-compartment model. All parameters were expressed as mean $\pm$ standard deviation (SD). The *t*-test (Prime 5 statistical software) was used to compare the pharmacokinetic parameters of each group. $P < 0.05$ was considered to be statistically significant.

3. Results and discussion

3.1 Optimization of sample procedures

In order to obtain the optimum extraction and preconcentration of butoconazole enantiomers from plasma and tissues, different sample pretreatment methods, including solid phase extraction (SPE), liquid–liquid extraction (LLE) and protein precipitation (PPT), were investigated in this study. For each procedure, the plasma and tissue samples containing butoconazole enantiomers with three QC concentrations were assessed.

First, the PPT method was investigated for the extraction of butoconazole enantiomers from plasma and tissue samples. The results indicated that a significant matrix effect was observed when using methanol or acetonitrile as a precipitation solvent. Thus, the PPT method was abandoned in our present study. In terms of the LLE method, according to the available literature,[3] methyl *tert*-butyl ether was effective in extracting butoconazole enantiomers from plasma with recoveries of above 79.43%. Additionally, we investigated three types of extraction solvents, including diethyl ether–dichloromethane (60 : 40), ethyl acetate and *n*-hexane-isopropanol (95 : 5). The results showed that, in comparsion with ethyl acetate and diethyl ether–dichloromethane (60 : 40), higher extraction recovery and a lower matrix effect were observed when using *n*-hexane–isopropanol (95 : 5) as the extraction solvent (recoveries >85.5%). Consequently, the volume of 2 mL of *n*-hexane–isopropanol (95 : 5) was selected as

Table 1 Recovery results for butoconazole enantiomers in rat plasma after using SPE and LLE preparation procedures

	Recovery (%, mean ± SD)			Recovery (%, mean ± SD)	
Liquid–liquid extraction (LLE)	(+)-Butoconazole	(−)-Butoconazole	Solid-phase extraction (SPE)	(+)-Butoconazole	(−)-Butoconazole
n-Hexane–isopropanol (95 : 5)	85.5 ± 4.5	86.3 ± 4.9	Cleanert C_{18}	89.2 ± 4.0	90.3 ± 5.9
Ethyl acetate	76.4 ± 4.0	75.3 ± 5.1	Cleanert NH_2	50.8 ± 3.4	49.3 ± 4.2
Diethyl ether–dichloromethane (60 : 40)	72.0 ± 4.1	72.8 ± 4.4	Cleanert PCX	42.0 ± 1.9	41.8 ± 2.0

the final extraction solvent for the LLE process. The overall extraction recovery results of the butoconazole enantiomers in rat plasma (mean ± SD) are summarized in Table 1.

During the development of the SPE extraction procedure, several different cartridges were thoroughly investigated, including Cleanert C_{18} cartridge (Agela, 500 mg, 3 mL), Cleanert NH_2 cartridge (Waters, 500 mg, 3 mL) and Cleanert PCX cartridge (Waters, 500 mg, 3 mL). All the three cartridges were checked under the same experimental conditions: the biological sample (200 μL of blank rat plasma or tissue homogenate) was taken with the addition of 20 μL of *rac*-butoconazole working solution; 3 mL of pure water was used as the washing solution and elution was performed using 3 mL of acetonitrile. The results showed that, after optimization, the reversed phase cartridge (Cleanert C_{18}) offered more effective butoconazole extraction than NH_2 and PCX cartridges (>89.2 for Cleanert C_{18}, >41.8% for Cleanert PCX, >49.3% for Cleanert NH_2). In summary, the SPE methodologies with C_{18} cartridges offered better extraction of the butoconazole enantiomers from plasma and tissue samples than with the tested LLE procedures. The detailed description of the developed SPE procedure with C_{18} cartridges chosen as the most optimal for the preparation of rat plasma and tissues is presented in the section "Sample Preparation". It should be noted that the developed C_{18} cartridge based SPE procedure provided higher recoveries of butoconazole than the previously reported data,[3–5] and it was the first report on the extraction of butoconazole enantiomers from plasma and tissues. In addition to providing higher recoveries, the samples were considered to be cleaner after SPE treatment, especially for the precious chiral columns that helped to prolong the chiral column life.

3.2 Optimization of mass spectrometry conditions

To develop a sensitive and stable mass spectrometry analytical method, the signal intensities of the precursor and fragment ions were investigated in positive and negative ionization modes. The results showed that a greater signal intensity and higher sensitivity for butoconazole and IS were observed in the positive ionization mode with the ESI source. Then, the standard solutions of *rac*-butoconazole and IS at a concentration of 100 ng mL^{-1} were directly infused into the mass spectrometry method to optimize the MRM conditions. In the Q1 full scan mode, the protonated precursor $[M + H]^+$ ions at *m*/*z* 413.15 and 388.26 were observed for butoconazole and IS, respectively. A stable and satisfactory signal-to-noise ratio (S/N) of butoconazole and IS was obtained for the fragment ions at *m*/*z* 165.01 and 133.11, respectively. Therefore, the predominating ion transitions at *m*/*z* 413.15 > 165.01 for the butoconazole enantiomers and *m*/*z* 388.26 > 133.11 for IS were selected in the following quantitative analysis. Other MRM parameters like the cone voltage and collision energy were also selected according to the MS response of the analyts. Satisfactory MS responses of butoconazole and IS were achieved when the cone voltages were set at 25 and 20 V, and the collision energies were set at 12 and 10 eV, respectively.

3.3 Optimization of the chiral separation

Generally, most chiral drugs can be separated with the chiral stationary phase in the normal phase. However, the normal phase mode was improper for the combination with a mass spectrometry detector due to the presence of a high proportion of *n*-hexane.[11,13] Therefore, the reversed phase mode was selected in the present study. The selection of chiral columns was supposed to be the most important factor affecting chiral separation.[14,15] Based on the literature reported,[16] three polysaccharide-based columns (Chiralpak IA, Chiralpak IB and Chiracel OD-RH) and the Chiralpak AGP column were tested to separate the butoconazole enantiomers under reversed phase conditions. It was observed that only the Chiralpak IC column showed superior enantioselectivity and less analysis time toward *rac*-butoconazole ($R_s \geq 2.86$ and $t_R < 15$ min). For Chiralpak AGP, the analysis time of butoconazole for complete chiral separation was about 25 min. The prolonged retention time was not conducive to the high-throughput biological sample analysis. However, the other two columns showed poor chiral recognition capability towards the butoconazole enantiomers although different mobile phase compositions and additives were investigated. Therefore, the Chiralpak IC column was finally chosen for the following study.

The effect of the type and content of organic modifiers on the chiral separation was evaluated. As shown in Table S1 (ESI†), the t_R and *R*s values of the butoconazole enantiomers increased obviously with the decreasing content of the modifier (methanol or acetonitrile). Compared with methanol/water as an eluent, the acetonitrile/water mobile phase provided complete resolution of the butoconaozle enantiomers and within less analysis time. Moreover, acetonitrile as an organic modifier could result in a better peak shape and higher column efficiency. The addition of acidic additives (0–0.2% of formic acid) and buffer solutions (0–10 mM of ammonium acetate) was also evaluated to achieve a satisfactory chiral separation. The results showed that the use of 10 mM ammonium acetate buffer in the mobile phase could greatly improve the enantioseparation of the butoconazole enantiomers. Accordingly, after a comprehensive

consideration of the resolution, retention time and peak shape, the Chiralpak IC column with the mobile phase of acetonitrile/10 mM aqueous ammonium acetate (90 : 10, v/v) was chosen as the optimal chromatographic condition for enantioseparation of the butoconazole enantiomers.

3.4 Method validation

3.4.1 Specificity and carry-over. Assay selectivity is demonstrated by the absence of interfering peaks at the retention times of (−)-butoconazole, (+)-butoconazole and IS in the extracted blank plasma and tissue samples. Fig. 3 shows the typical chromatograms of the blank samples (A1-plasma, B1-heart), blank samples spiked with *rac*-butoconazole at the LLOQ level (0.5 ng mL^{-1} per enantiomer for plasma, 1.0 ng mL^{-1} per enantiomer for heart; A2-plasma, B2-heart), and actual rat plasma samples obtained at 6.0 h (heart at 24 h) after transdermal administration of 10 mg kg^{-1} 2% *rac*-butoconazole nitrate cream to female Sprague-Dawley rats (C1-plasma, C2-heart). As seen from Fig. 3, there were no endogenous interference peaks eluted at the retention times of (−)-butoconazole, (+)-butoconazole and IS in the blank plasma and tissues. Meanwhile, the carry-over of butoconazole and IS met the standard requirements. The results suggested that the carry-over effect was negligible in the LC-MS/MS system, and the ULOQ samples had no effect on the accurate determination of the next sample.

3.4.2 Linearity and LLOQ. The calibration curves showed excellent linearity over the range of 0.5–250 ng mL^{-1} for (−)- and (+)-butoconazole in plasma and tissues with a correlation coefficient (R^2) greater than 0.991. Under the stated conditions, the proposed method offered an LLOQ of 0.5 ng mL^{-1} for each enantiomer in the plasma sample (1.0 ng mL^{-1} per enantiomer in kidneys, liver and heart, 2.0 ng mL^{-1} per enantiomer in lungs) with an acceptable accuracy and precision.

3.4.3 Accuracy and precision. The results of precision and accuracy at three QC concentration levels analyzed in six replicates on three consecutive days are presented in Table 2. The RSD values of the intra- and inter-day precision for (−)-butoconazole were <10.3%, whereas the RE values of accuracy varied from −8.0 to 9.1%. For (+)-butoconazole, the RSD values of the intra- and inter-day precision were within 11.5%, while the RE values of accuracy ranged from −6.8 to 7.2%. These data fulfilled the desired criteria, demonstrating that this method was feasible and reproducible for the measurement of *rac*-butoconazole enantiomers in rat plasma and tissues.

3.4.4 Extraction recovery and the matrix effect. The recovery values of (−)- and (+)-butoconazole changed from 91.1 to 105.8% and from 90.4 to 104.6% at three QC concentrations in rat plasma and tissues with all RSD values within 11.2%. The extraction recovery of IS was 93.6%, and RSD was 7.8%. These results confirmed the validity of the extraction procedure for sample preparation to obtain reproducible and reliable quantitative results for the analytes and IS. For (−)- and (+)-butoconazole, the NMF was in the range of 101.4–108.2% and 102.9–107.8%, respectively, and RSD was below 8.2%. Accordingly, the ion suppression or enhancement from the plasma and the tissue matrix was negligible in this work.

3.4.5 Stability. The results of the stability studies are presented in Table 3. No significant degradation of analytes or IS was observed in all the conditions as described in Section "2.7.5". As per the stability conditions described above, the accuracy in plasma and tissues ranged from −11.2 to 10.2% and from −10.3 to 9.6% for (−)- and (+)-butoconazole, respectively, and RSD was lower than 11.8%.

3.5 Application to the enantioselective pharmacokinetic study

The newly developed LC-ESI-MS/MS method was successfully applied to the enantioselective pharmacokinetic study of (−)- and (+)-butoconazole after transdermal administration of 10 mg kg^{-1} 2% *rac*-butoconazole nitrate cream to female Sprague-Dawley rats. As far as we know, this is the first enantioselective assay for butoconazole enantiomers *in vivo*. The mean plasma concentration–time profiles of the butoconazole enantiomers quantified within 48 h are presented in Fig. 4, and the corresponding pharmacokinetic parameters are summarized in Table 4. The pharmacokinetic parameters between the two butoconazole enantiomers were compared by paired sample

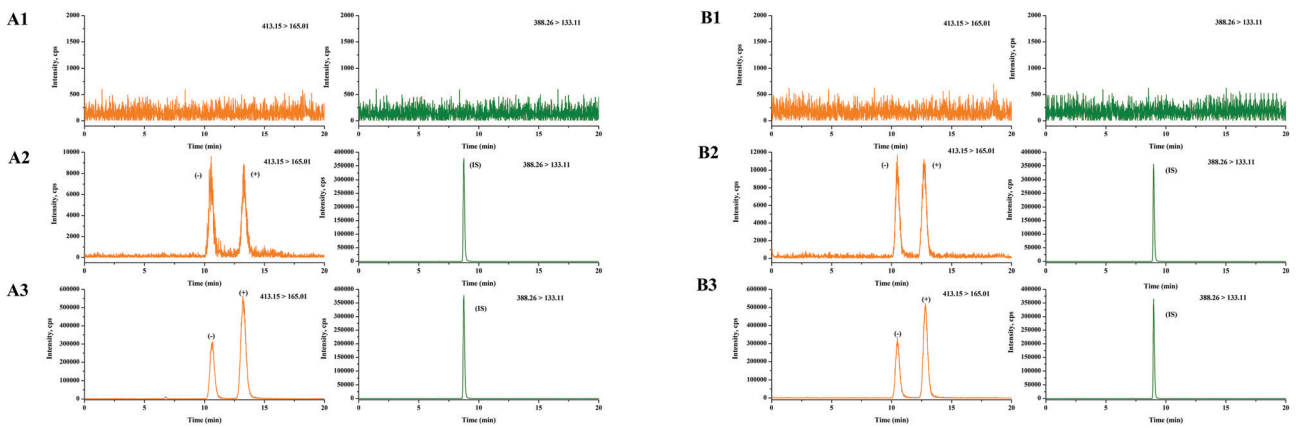

Fig. 3 Representative MRM chromatograms of the blank plasma (A1) and blank heart homogenate (B1); blank plasma (A2) and blank heart homogenate (B2) spiked with *rac*-butoconazole at the LLOQ level; real plasma (A3) and real heart homogenate (B3) collected at 6 h for plasma and 24 h for heart after transdermal administration of 10 mg kg^{-1} 2% *rac*-butoconazole nitrate cream to female Sprague-Dawley rats.

Table 2 Intra- and inter-day accuracy and precision of the analysis method in spiked rat plasma and tissues at three QC levels

			Intra-day (n = 6)			Inter-day (n = 18)		
	Analytes	Nominal concentration (ng mL^{-1})	Calculated concentration (ng mL^{-1})	RE (%)	RSD (%)	Calculated concentration (ng mL^{-1})	RE (%)	RSD (%)
Plasma	(−)-Butoconazole	2.0	2.1 ± 0.1	−4.6	4.8	2.1 ± 0.2	5.9	9.5
		20.0	20.6 ± 0.8	4.8	3.9	21.4 ± 0.9	4.4	4.2
		40.0	42.2 ± 1.1	6.1	2.6	41.9 ± 1.9	5.5	4.5
	(+)-Butoconazole	2.0	2.0 ± 0.1	−5.7	5.0	2.2 ± 0.2	6.7	9.1
		20.0	21.1 ± 1.2	2.9	5.6	20.8 ± 0.9	3.4	4.3
		40.0	41.0 ± 1.6	5.4	3.9	42.7 ± 2.6	4.0	6.1
Kidneys	(−)-Butoconazole	2.5	2.4 ± 0.1	−6.6	4.2	2.5 ± 0.2	9.1	8.8
		50.0	52.1 ± 1.4	5.0	2.7	48.9 ± 2.2	−4.4	4.5
		200.0	205.8 ± 4.4	1.9	2.1	197.6 ± 7.2	−2.9	3.6
	(+)-Butoconazole	2.5	2.5 ± 0.1	−4.2	4.0	2.4 ± 0.2	6.8	8.3
		50.0	51.8 ± 2.5	4.4	4.8	49.7 ± 3.2	−3.7	6.4
		200.0	208.8 ± 5.2	3.9	2.5	195.5 ± 9.6	−1.8	4.9
Liver	(−)-Butoconazole	2.5	2.4 ± 0.2	−5.5	8.3	2.5 ± 0.2	6.7	8.0
		50.0	51.1 ± 1.8	4.6	3.5	51.1 ± 2.1	4.8	4.1
		200.0	197.8 ± 6.5	−1.9	3.3	203.0 ± 5.3	5.9	2.6
	(+)-Butoconazole	2.5	2.5 ± 0.2	−3.8	8.0	2.6 ± 0.2	7.2	7.7
		50.0	50.9 ± 1.5	5.7	2.9	52.0 ± 2.8	3.8	5.4
		200.0	198.5 ± 5.4	−3.1	2.7	205.5 ± 3.9	1.9	1.9
Heart	(−)-Butoconazole	2.5	2.9 ± 0.3	6.4	10.3	2.5 ± 0.2	−5.0	8.0
		40.0	42.2 ± 2.8	3.8	6.6	41.9 ± 3.0	2.7	7.2
		80.0	79.1 ± 3.0	−1.9	3.8	83.3 ± 2.5	4.9	3.0
	(+)-Butoconazole	2.5	2.7 ± 0.2	5.8	7.4	2.4 ± 0.2	−3.8	8.3
		40.0	41.8 ± 2.4	4.4	5.7	44.1 ± 3.9	3.0	8.8
		80.0	78.2 ± 2.3	−2.2	2.9	82.0 ± 2.2	6.1	2.7
Lungs	(−)-Butoconazole	8.0	8.2 ± 0.6	5.9	7.3	7.8 ± 0.8	−8.0	10.2
		50.0	52.5 ± 2.2	−1.9	4.2	53.0 ± 3.1	3.9	5.8
		160.0	163.0 ± 4.4	2.4	2.7	164.8 ± 3.8	2.2	2.3
	(+)-Butoconazole	8.0	8.3 ± 0.8	6.6	9.6	7.9 ± 0.9	−6.8	11.5
		50.0	54.4 ± 2.8	−3.0	5.1	51.9 ± 4.0	5.2	7.7
		160.0	165.3 ± 5.5	1.7	3.3	163.5 ± 4.4	1.0	2.7

T-tests. Statistically, a significant difference existed between the parameters of (−)- and (+)-butoconazole when $P < 0.05$.

As can be seen in Fig. 4, the concentrations of both enantiomers slowly increased following transdermal absorption and reached the maximum plasma concentration after 6 h, which is in accordance with the literature data.[3] The plasma concentration of the (+)-butoconazole enantiomer in rat plasma was always higher than that of its antipode. By comparing the drug concentration–time curves of the two enantiomers more precisely, we could find that the mean C_{max} value of (+)-butoconazole was 1.75 times greater than that of (−)-butoconazole, and the $AUC_{0-\infty}$ value was 1.72-fold higher than that of (−)-butoconazole, which repeatedly corroborated that (+)-butoconazole presented enhanced pharmacodynamic activity compared with (−)-butoconazole. The results are consistent with the previous literature data of chiral antifungal drugs ketoconazole and fenticonazole,[7,8] in which the plasma concentration of the (+)-isomer form was almost higher than that of its antipode. Except for the difference in the absorption phase, the CLz/F value of (−)-butoconazole was 1.68 times higher than that of the (+)-isomer (seen from Table 4). This results indicated that the elimination of (−)-butoconazole was much faster than that of (+)-butoconazole after transdermal administration, resulting in the enrichment of (+)-butoconazole in rat plasma. Briefly, these results indicated the substantial stereoselectivity on the pharmacokinetics of the butoconazole enantiomers in female Sprague-Dawley rats.

3.6 Application to enantioselective tissue distribution

After the rats were transdermally administrated with 10 mg kg^{-1} 2% *rac*-butoconazole nitrate cream, the drug concentrations in different tissues were calculated according to the tissue weight. The results are shown in Fig. 5. As seen, (−)- and (+)-butoconazole were found to be widely distributed in tissues. The liver and kidneys were the two main organs of distribution, followed by the lungs and heart. This result also demonstrated that the drug may have potential hepatotoxicity, which will be further studied in the next experiment. Compared with the tissues mentioned above, the distribution concentration in the brain was too low for precise quantification, indicating that butoconazole might not easily pass the blood–brain barrier. As seen from Fig. 5, the tissue distribution between the (−)- and (+)-isomers differed in enantioselectivity for each tissue and each time point. The peak concentration time appeared at 10 h, except for the heart at 24 h, and the concentration of (+)-butoconazole in the kidneys, liver, heart and lungs was 1.4–2.0, 1.2–1.9, 1.5–2.2 and 1.3–1.7 times higher than that of (−)-butoconazole, respectively. Moreover, the (+)-butoconazole concentration was always higher than that of (−)-butoconazole, indicating that (+)-butoconazole was tend to exist in various tissues leading to a slower metabolism than compared to (−)-butoconazole. These results are consistent with the pharmacokinetic parameters reported above. Besides, the higher

Table 3 Stability results for (−)- and (+)-butoconazole under various storage conditions (n = 6)

	Analytes	Nominal concentration (ng mL^{-1})	Room temperature for 12 h		On the autosampler tray 4 °C for 24 h		−80 °C for 30 days		Three freeze–thaw cycles	
			RE (%)	RSD (%)	RE (%)	RSD (%)	RE (%)	RSD (%)	RE (%)	RSD (%)
Plasma	(−)-Butoconazole	2.0	−7.0	10.3	−5.4	9.7	9.3	8.8	7.4	10.2
		20.0	5.5	7.6	−3.8	6.6	−4.8	6.2	5.3	7.4
		40.0	4.8	5.0	2.9	4.0	3.6	1.9	5.0	2.9
	(+)-Butoconazole	2.0	−6.6	9.8	−6.0	10.4	9.0	8.5	8.0	9.3
		20.0	5.1	7.2	−3.5	5.8	−5.5	5.9	5.5	6.8
		40.0	5.0	4.7	3.3	4.3	3.3	1.5	4.3	1.8
Kidneys	(−)-Butoconazole	2.5	8.6	7.8	−9.6	11.8	6.6	8.2	7.7	10.1
		50.0	8.8	4.0	7.6	5.6	7.5	5.0	8.0	6.6
		200.0	−1.9	0.9	1.9	2.2	2.0	1.7	−2.1	2.9
	(+)-Butoconazole	2.5	9.2	8.2	−9.8	10.8	7.1	8.5	8.9	9.4
		50.0	8.5	4.4	6.6	6.0	8.0	4.8	8.2	6.3
		200.0	−3.0	1.1	1.5	1.8	1.8	1.2	−1.7	3.2
Liver	(−)-Butoconazole	2.5	−11.2	8.8	−7.7	6.2	10.2	8.2	8.8	6.1
		50.0	6.8	4.0	6.2	2.9	−6.6	8.9	7.0	6.7
		200.0	1.1	1.9	5.0	0.8	−4.8	1.5	3.3	2.2
	(+)-Butoconazole	2.5	−10.3	9.2	−8.0	7.0	9.6	7.8	8.1	6.8
		50.0	6.2	4.4	6.5	2.2	−5.7	9.4	7.4	6.0
		200.0	1.7	2.2	4.3	1.2	−4.5	1.0	2.9	1.8
Heart	(−)-Butoconazole	2.5	−6.0	9.5	7.7	10.8	−9.9	8.9	6.4	8.5
		40.0	2.9	4.8	2.9	7.8	8.0	8.8	5.9	8.8
		80.0	1.4	1.2	1.9	5.3	−3.5	5.7	2.2	2.0
	(+)-Butoconazole	2.5	−7.1	10.7	8.2	11.1	−9.2	8.5	6.9	8.7
		40.0	2.8	6.6	4.0	7.0	7.4	9.0	5.5	9.1
		80.0	0.9	1.7	2.1	4.9	−3.0	5.5	1.8	1.7
Lungs	(−)-Butoconazole	8.0	−8.8	10.3	4.7	8.8	−6.6	9.1	8.0	9.5
		50.0	6.7	5.5	5.8	4.0	−4.8	6.6	7.8	9.1
		160.0	2.0	2.9	1.5	2.8	0.5	1.8	2.3	1.8
	(+)-Butoconazole	8.0	−8.4	9.9	5.0	8.3	−7.2	8.2	9.1	10.5
		50.0	6.2	5.8	5.5	4.2	−4.3	6.0	7.7	9.8
		160.0	1.9	3.1	2.1	2.0	0.9	1.5	2.0	1.7

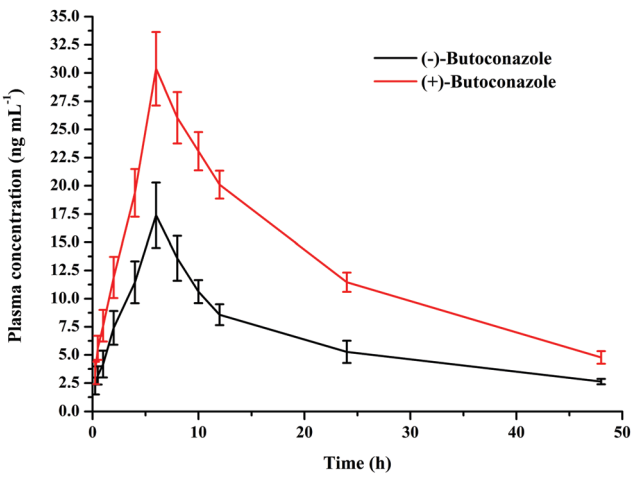

Fig. 4 Plasma concentration–time curves obtained after transdermal administration of 10 mg kg^{-1} 2% *rac*-butoconazole nitrate cream to female Sprague-Dawley rats (n = 6).

Table 4 Pharmacokinetic parameters (mean ± SD) of the butoconazole enantiomers in female Sprague-Dawley rats following transdermal administration at a dose of 10 mg kg^{-1} (n = 6)

Parameters	(+)-Butoconazole	(−)-Butoconazole	(+)/(−)-Butoconazole
C_{max} (ng mL^{-1})	30.40 ± 5.33	17.40 ± 4.30	1.75 ± 0.12**
T_{max} (h)	6.00	6.00	1.00
$t_{1/2}$ (h)	17.74 ± 1.91	17.22 ± 1.60	1.04 ± 0.38
$AUC_{0-48\,h}$ (ng h mL^{-1})	501.21 ± 28.86	298.03 ± 36.35	1.68 ± 0.10***
$AUC_{0-\infty}$ (ng h mL^{-1})	603.40 ± 51.55	348.50 ± 41.87	1.72 ± 0.25 **
MRT_{0-48} (h)	17.41 ± 2.33	15.36 ± 2.43	1.13 ± 0.05
CLz/F (L h^{-1} kg^{-1})	17229 ± 2494	28998 ± 1383	0.59 ± 0.08***

Values significantly (**$p < 0.05$, ***$p < 0.01$) different from the concentration of (−)-butoconazole; C_{max}, the maximum plasma concentration; T_{max}, the time to reach the maximum plasma concentration; $t_{1/2}$, half-life of elimination; AUC_{0-48}, area under the plasma concentration curve from 0 to 48 h; $AUC_{0-\infty}$, area under the plasma concentration curve from 0 to infinity; MRT_{0-48}, the mean retention time from 0 to 48 h; CLz/F, apparent clearance.

concentration of (+)-butoconazole in plasma might be the cause of the differences in the enantioselective distribution between (−)- and (+)-butoconazole. Additionally, the concentration of the butoconazole enantiomers in all tissues was low after 48 h transdermal administration, suggesting that the drug did not accumulate in tissues for a long time. By summarizing the results of pharmacokinetics and tissue distribution, significant enantioselective differences between (−)- and (+)-butoconazole were found to exist not only in the stage of pharmacokinetics but also in tissue distribution. Such results are of great significance for the further study of the behavior of butoconazole

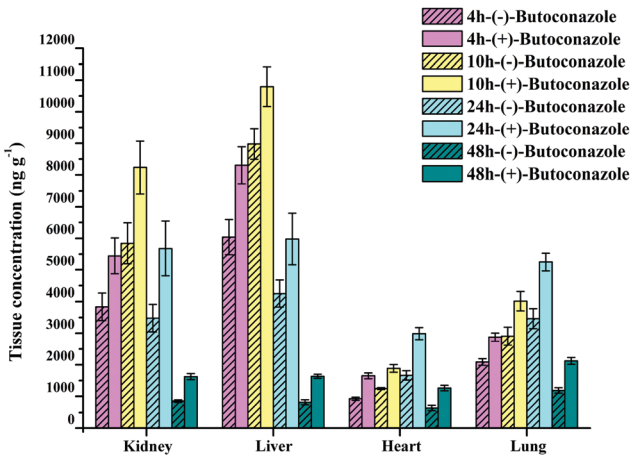

Fig. 5 Mean tissue distribution of butoconazole in kidneys, liver, heart and lungs at 4 h, 10 h, 24 h and 48 h after transdermal administration of 10 mg kg^{-1} 2% *rac*-butoconazole nitrate cream to female Sprague-Dawley rats (n = 24).

enantiomers *in vivo* and the potential development of the single isomer drug of butoconazole.

4. Conclusion

In the present study, a novel, sensitive, reliable and enantioselective LC-ESI-MS/MS method using the Chiralpak IC column for the determination of (−)- and (+)-butoconazole was successfully established and validated. Biological samples (plasma and tissues) were prepared by a rapid and effective SPE procedure with the C_{18} cartridge, which reduced the time required for the sample preparation and provided more clean plasma and tissue samples and can be considered as an interesting analytical tool in comparison with previously reported methods. After validation, the developed method was successfully applied to the study on the enantioselective pharmacokinetics and tissue distribution of butoconazole enantiomers in female SD rats. The pharmacokinetic outcomes revealed that (+)-butoconazole displayed prominently higher C_{max} and AUC values, and a slower metabolism rate than the parameters of the (−)-enantiomer in rat plasma. For the tissue distribution, the liver and kidney were the two main organs of distribution, and the consequence of enantioselective tissue distribution differences between the two butoconazole enantiomer was as follows: heart > kidneys > liver > lungs. This is the first experimental evidence of the enantioselective behavior of butoconazole *in vivo*.

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Author contributions

Siman Ma: conceptualization, methodology, investigation, and writing – original draft preparation. Guoxian Guo: software: validation. Lina Wang: conceptualization, software, and writing – review and editing. Jia Yu: conceptualization, writing – review and editing. Xingjie Guo: conceptualization, resources, writing – review and editing.

Conflicts of interest

There are no conflicts to declare.

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