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Short communication

Determination of butenafine hydrochloride in human plasma by liquid chromatography electrospray ionization-mass spectrometry following its topical administration in human subjects

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ABSTRACT

An HPLC/MS/MS method for determination of butenafine hydrochloride in human plasma with testosterone propionate as the internal standard (IS) was developed and validated. Plasma samples were extracted with an n-hexane/diethyl ether (1:2, v/v) mixture and separated using a C_{18} column by a gradient elution with the mobile phase containing acetonitrile and 5 mM ammonium acetate buffer. Quantification was performed using multiple reaction monitoring (MRM) mode with transition of m/z 318.4 → 141.0 for butenafine hydrochloride and m/z 345.5 → 97.0 for testosterone propionate (IS). This method was validated in terms of specificity, linearity, precision, accuracy, and stability. The lower limit of quantification (LLOQ) of this method was 0.0182 ng/ml and the calibration curve was linear over the 0.0182–1.82 ng/ml. The intra- and inter-run coefficient of variance was less than 11.53% and 10.07%, respectively. The samples were stable under all the tested conditions. The method was successfully applied to study the pharmacokinetics of butenafine hydrochloride in healthy Chinese volunteers following its topical administration.

1. Introduction

Butenafine hydrochloride, N-(4-tert-butylbenzyl)-N-methyl-1-naphthalene methylamine hydrochloride, is a benzylamine derivative with chemical structure and mechanism similar to allylamine antifungals. It exhibits potent fungicidal activity particularly against dermatophytes, aspergilli, dimorphic and dematiaceous fungi. Topical butenafine cream (1%) has been reported to be effective for the treatment of tinea pedis, tinea corporis and tinea cruris for short term therapy [1]. When given topically, high concentration of butenafine hydrochloride was found in epidermis, including the horny keratinised layer, which could subsequently penetrate to the deeper layers of the dermis via sebaceous glands and hair follicles [2].

For a topical product like butenafine hydrochloride, there is no standard approach specified by the regulatory agency for evaluation of bioavailability and bioequivalence [3]. However, the plasma concentration of the drug can be utilized to evaluate the safety and the pharmacokinetic process in vivo. The plasma concentration–time profile could be used as an indication of clinical safety, although the systemic availability may be irrelevant to local cutaneous bioavailability. For most topically administered drugs, usually only trace amount of these drugs are present in serum or plasma and their concentrations are usually too low to be detected by conventional assay techniques, such as UV spectroscopy or HPLC-DAD method. The HPLC chromatographic methods have been reported for determination of butenafine hydrochloride in matrix such as cream formulation [4,5], but these methods are not sensitive enough (LLOQ was 2.5–100 μg/ml) to determine the plasma concentration. A previous study was performed to examine the potential systemic exposure of butenafine following administration of Mentax (butenafine HCl l% cream) using a dose higher than the one clinically recommended [6]. However, this approach is still not suitable due to insufficient sensitivity in the assay. Thus in this study, a sensitive LC–MS/MS method to determine the plasma concentration of butenafine hydrochloride was developed and applied to a pharmacokinetic study in human subjects following its topical application at normal therapeutic dose.

2. Experimental

2.1. Reagents and chemicals

Butenafine hydrochloride 1% cream was manufactured by Kunming Dihon Pharmaceutical Co., Ltd. (Kunming, China). Butenafine hydrochloride standard was obtained from the National Institute

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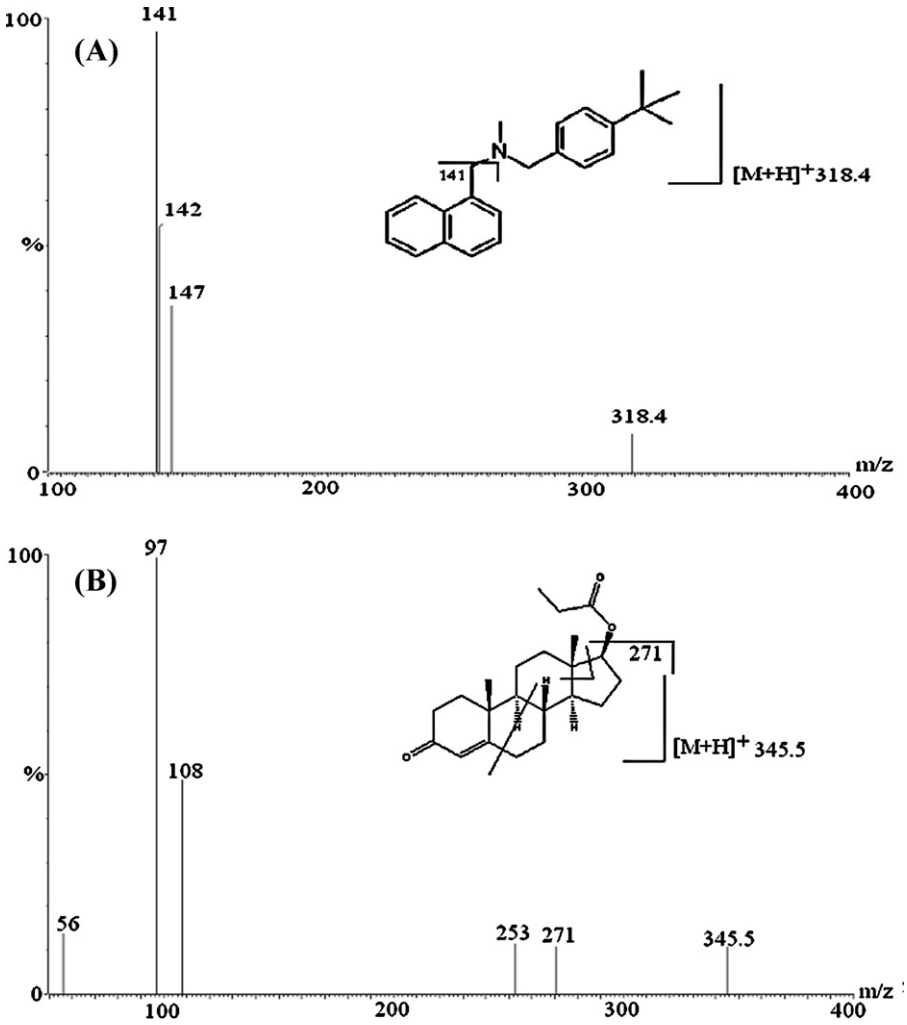

Fig. 1. Chemical structures with fragmentation and produce ion spectrum of $[M+H]^+$ of butenafine hydrochloride (a) and testosterone propionate (b) (IS).

for the Control of Pharmaceutical and Biological Products (Beijing, China). Testosterone propionate was purchased from Dr. Ehrenstorfer GmbH (Augsburg, Germany). Diethyl ether of analytical grade was purchased from Tianjing Zhiyuan Chemical Reagent Co., Ltd. (Tianjing, China). n-Hexane, acetonitrile and ammonium acetate of HPLC grade were obtained from TEDIA (Fairfield, OH, USA). Water used throughout the experiment was generated by a Milli-Q academic water purification system (Milford, MA, USA). Human plasma was obtained from the Blood Center of Chengdu (Chengdu, China).

2.2. Preparation of standard calibration and QC samples

The stock solution of butenafine hydrochloride with concentration of 0.728 mg/ml was prepared by dissolving appropriate amount of butenafine hydrochloride standard in acetonitrile and further diluted using acetonitrile to generate a serial of concentrations ranged from 0.364 to 36.4 ng/ml as the working solution. The stock solution of testosterone propionate (internal standard, IS) was prepared in acetonitrile to generate the concentration of 0.928 mg/ml and further dilute to 0.928 μg/ml as the working solution. All the solutions were stored at 4 °C and tested to be stable for at least 50 days.

Calibration standards as well as the quality control (QCs) samples at concentrations of 0.0182, 0.0364, 0.0728, 0.182, 0.364, 0.728 and 1.82 ng/ml were prepared by spiking the corresponding working solutions into 1 ml of blank plasma.

2.3. Sample processing

For analysis of the real clinical samples, 50 μl of acetonitrile and 50 μl of IS working solutions were added to 1 ml plasma and mixed well by vortexing briefly. Liquid–liquid extraction was then performed by addition of 4 ml of an n-hexane/diethyl ether (1:2, v/v) mixture, followed by vortex extraction for 3 min (IKA Votex Genius 3 Votex, Germany). After centrifugation at 12,000 rpm for 5 min, the upper organic layer was transferred into another neat tube and evaporated to dryness completely at 40 °C under a stream of nitrogen (Turbovap Zymark, Hopkinton, MA, US). The dry residue was reconstituted with 100 μl of mobile phase and a 10 μl of aliquot was injected to HPLC–MS/MS system for analysis.

2.4. HPLC–MS/MS analysis

LC–MS/MS analysis was performed on a Waters (Quattro Premier XE MircoMass) HPLC (Waters 2695)–MS/MS system. A SymmetryShield™ RP18 column (150 mm × 2.1 mm, 5 μm) was used for the chromatographic separation. The column was maintained at 30 °C. A gradient elution was carried out using a mobile phase containing acetonitrile (A) and 5 mM ammonium acetate

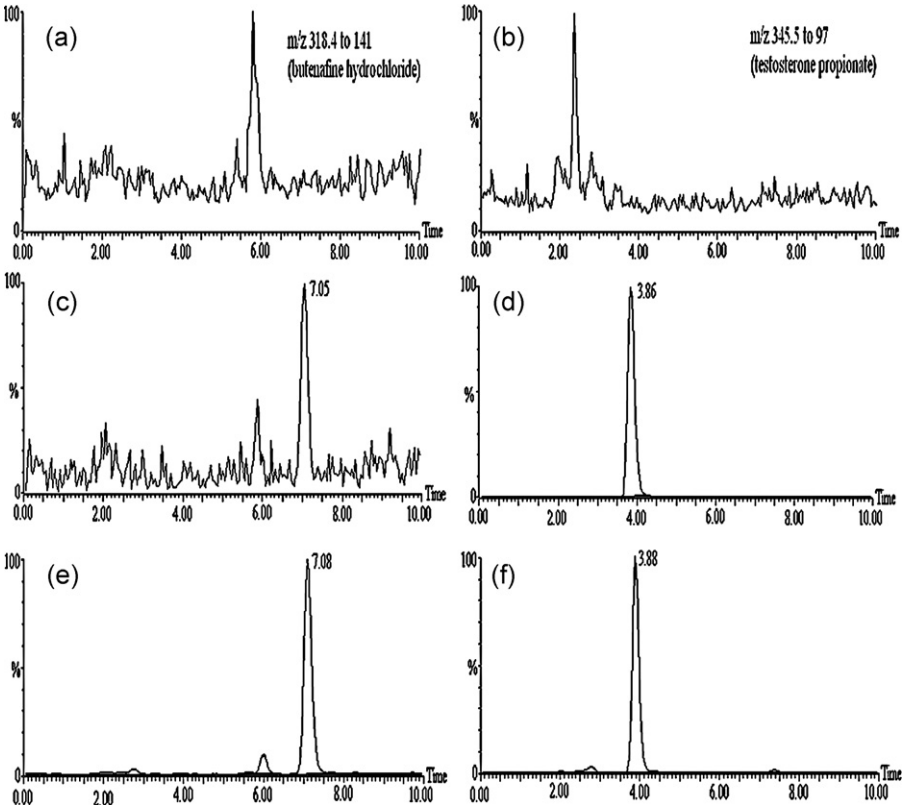

Fig. 2. MRM chromatogram of (a) and (b): blank plasma; (c) and (d) 0.0182 ng/ml of butenafine hydrochloride with its IS (46.4 ng/ml); (e) and (f) a plasma sample obtained from a subject at 24 h post dosing (concentration determined was 0.16 ng/ml) with its IS (46.4 ng/ml).

solution (B) at a flow rate of 0.2 ml/min. The elution was started at 85% of A. Then the percentage of A was changed linearly to 100% in 3 min and hold for 3.5 min. Afterwards, the ratio was changed back to the original 85% in 0.1 min and equilibrated for another 3.4 min. The entire run time was 10 min. The ESI-MS/MS was carried out using nitrogen to assist nebulization. The capillary voltage was set at 3000 V. Desolvation gas (nitrogen) was heated to 350 °C and delivered at a flow-rate of 450 l/h, and the source temperature was 110 °C. The cone voltage was set at 20 V, and the cone gas flow was 50 l/h. Quantification was performed using multiple reaction monitoring (MRM) mode with transition of m/z 318.4 → 141.0 for butenafine hydrochloride and m/z 345.5 → 97.0 for testosterone propionate (IS). During the data acquisition phase, the delta potential of the electron multiplier (EMV) was set to 650 V.

2.5. Validation of the method

2.5.1. Specificity and selectivity

Specificity was evaluated in terms of the endogenous interference by analyzing blank human plasma samples from 6 different individuals. Blank matrix samples spiked only with IS or butenafine hydrochloride were analyzed to assess potential interference that may affect the butenafine hydrochloride or IS.

2.5.2. Sensitivity and linearity

The standards were prepared by spiking blank human plasma with the working solutions of butenafine hydrochloride. The calibration curve was constructed by plotting the peak area ratios (R) of butenafine hydrochloride to the IS versus the concentrations (C) of butenafine hydrochloride and fitted by weighed least squares linear regression (weighing factor was $1/C^2$). The LLOQ was defined as the lowest analytical concentration at which the analyte peak should be identifiable, discrete, reproducible with a precision within 20% and accuracy of 80–120%, and the signal–noise ratio of more than 5:1.

2.5.3. Precision and accuracy

Intra-day, inter-day precision and accuracy were determined by analyzing QC samples at three different concentrations ($n = 5$) on three different days. The precision was defined as the relative standard deviation (RSD) of QC sample concentrations determined at 5 replicates, whereas accuracy was assessed as the percentage to the nominal concentration (%). The mean values should be within 15% of the nominal value except at LLOQ, which should not deviate more than 20%.

2.5.4. Extraction recovery and matrix effect

The extraction recoveries of butenafine hydrochloride at low, medium and high concentrations were determined by comparing the peak areas obtained from the spiked plasma with the extracted samples of blank plasma spiked with the standard solutions. It represented a recovery value that is not affected by the matrix. The absolute matrix effect for butenafine hydrochloride was evaluated by comparing the peak area of the analyte in the extracted blank plasma samples from five different drug-free volunteers.

2.5.5. Stability

Stability of the analytes were tested using three different QC sample concentrations under different experimental conditions including three freeze–thaw cycles, storage for 80 days at −20 °C, sitting in autosampler vials at 25 °C for 12 h and at room temperature for 8 h. A deviation less than 20% from the original sample was considered to be stable.

Table 1
Precision and accuracy of the developed method for the determination of butenafine hydrochloride in human plasma (data were based on assay of five replicates per day, on three different days).

Concentration (ng/ml)	Intra-day determination			Inter-day determination		
	Determined conc. (ng/ml)	Precision (%)	Accuracy (%)	Determined conc. (ng/ml)	Precision (%)	Accuracy (%)
0.0182	0.0170 ± 0.0020	11.53	93.20	0.0166 ± 0.0017	10.07	91.20
0.0364	0.0332 ± 0.0019	5.68	91.16	0.0332 ± 0.0026	7.73	91.15
0.182	0.168 ± 0.007	4.01	92.20	0.174 ± 0.011	6.42	95.66
0.728	0.750 ± 0.046	6.09	102.96	0.702 ± 0.054	7.74	96.50

2.6. Application of the method to a pharmacokinetic study

The assay was used to determine butenafine plasma concentration following transdermal application of 33 mg butenafine hydrochloride to 1650 cm^2 of the body surface area (on the upper back) in 10 healthy Chinese volunteers (5 male, 5 female). The clinical study protocol was reviewed and approved by the Ethics Committee of Xijing Hospital Affiliated to the Fourth Military Medical University of Chinese People's Liberation Army. All volunteers provided written informed consent to participate in the study according to the principles of the Declaration of Helsinki.

For these 10 volunteers, their mean age was 36.2 years (range: 30–40 years) with mean body weight 58.2 kg (range: 50–63 kg). Following overnight fasting, each volunteer received 33 mg butenafine hydrochloride topically. Standard meals were provided after 4 h post-dose. Blood samples were collected pre-dose and at 2, 4, 6, 8, 10, 14, 18, 24, 30, 36, 48, 60, 72, 96 and 120 h post-dose. The butenafine hydrochloride plasma concentrations were determined using the LC/MS/MS method described above. Model-independent pharmacokinetic parameters were calculated for butenafine hydrochloride. The maximum plasma concentration (C_{max}) and peak time (t_{max}) were noted directly by inspection of the concentration–time curve. The elimination rate constant (k_{el}) was calculated by semi-long linear regression of the terminal phase of plasma concentration–time curve. Elimination half-life ($t_{1/2}$) was calculated using the formula $t_{1/2} = 0.693/k_{el}$. The area under the plasma concentration–time curve AUC_{0-120} to the last measurable plasma concentration was calculated by the linear trapezoidal rule.

3. Results and discussion

3.1. Mass spectrometry

Analyte and internal standard responded best to positive ionization with the protonated ions $[M+H]^+$ being presented as major peaks for both compounds. Their product ion mass spectra are shown in Fig. 1. Multiple reaction monitoring (MRM) mode was used to identify the molecules by monitoring the transition of m/z from 318.4 to 141.0 for butenafine hydrochloride and 345.5 to 97.0 for IS, respectively.

3.2. Chromatography

The representative chromatograms of butenafine hydrochloride and IS in plasma samples are shown in Fig. 2. The chromatographic conditions were optimized to achieve high sensitivity, speed, and peak shape. The results showed that the ammonium acetate solution not only could improve peak shape of butenafine hydrochloride, but also increase the MS sensitivity via aiding protonation. So a concentration of 5 mM ammonium acetate was added in mobile phase. Since butenafine is a nonpolar molecule, high percentage of acetonitrile was used in the mobile phase to shorten the running time in HPLC. Under the optimum conditions, the retention time was 3.4 min for butenafine hydrochloride and 2.8 min for IS.

3.3. Assay validation

3.3.1. Specificity

The analysis of analyte and internal standard using the MRM function was highly selective. There was no interference or significant ion suppression from endogenous substances in the matrix. Representative chromatograms obtained from blank plasma, blank plasma spiked with analyte and internal standard and a plasma sample from a representative subject at 24 h post dosing are shown in Fig. 2.

3.3.2. Calibration curves and LLOQ

The calibration curves were linear in the concentration range 0.0182–1.82 ng/ml, $R = 0.002 + 3.528\,C$ (where the R is peak area ratio of butenafine to IS and C is the nominal concentration), $r = 0.9991$. The LLOQ was 0.0182 ng/ml. The high sensitivity of our method provided the capability of determining the concentrations of butenafine in human plasma up to 120 h after administration of a regular dose of 33 mg butenafine hydrochloride.

3.3.3. Precision and accuracy

The intra- and inter-day precision and accuracy of the assay are summarized in Table 1. Intra-day and inter-day precisions were 4.01–11.53% and 6.42–10.07%, respectively, with accuracy ranging from 91.16 to 102.96%.

3.3.4. Matrix and recovery

The mean extraction recoveries were 82.86 ± 5.58, 86.56 ± 5.55 and 89.21 ± 5.96% for the concentrations of 0.0364, 0.182 and 0.728 ng/ml, respectively. The mean extraction recovery was 77.92 ± 4.65% for IS. The absolute matrix effect was within the range of 92.69–106.58% for butenafine hydrochloride and 92.89–104.40% for IS. Thus the method was considered to be valid, due to the similar relative matrix effect and unobvious absolute matrix effect.

3.3.5. Stability

The analytes were stable in human plasma after storage for 80 days at −20 °C, or 8 h at room temperature with deviations < 7.56% from the baseline level. No significant degradation of butenafine hydrochloride was observed when kept in the auto-sampler for up to 12 h at room temperature (with a deviation less than 7.99% from the baseline level). The analytes were also found to be stable over three freeze–thaw cycles.

3.4. Application of the method

The mean plasma concentration–time curve of butenafine hydrochloride is shown in Fig. 3, and the main pharmacokinetic parameters are shown in Table 2. The current method provided sufficient sensitivity and reliability to carry out a pharmacokinetic study on butenafine based on sufficient sampling points after topical administration of a regular dose. This method is clearly an advantage over the previous method by Gibbs [6], which is much less sensitivity and convenient. Furthermore, the study required a dose considerably higher than that used clinically.

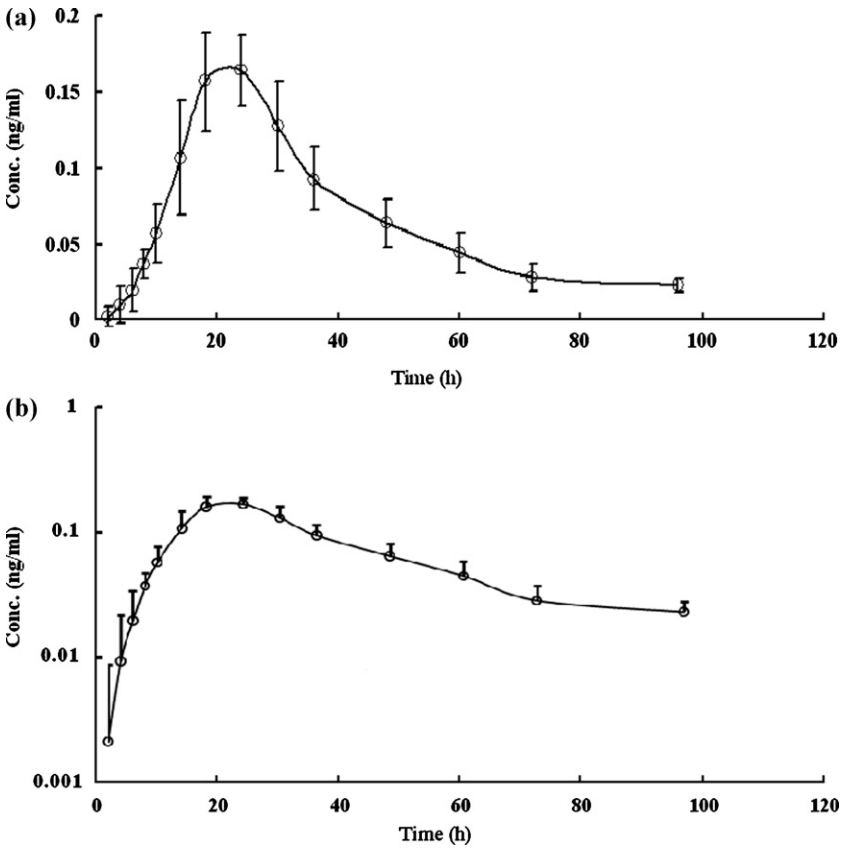

Fig. 3. Mean plasma concentration–time course (a) and semi-log scale (b) in 10 subjects following a transdermal drug delivery of 33 mg butenafine hydrochloride.

Table 2
Mean pharmacokinetic parameters of butenafine hydrochloride after transdermal drug delivery of 33 mg butenafine hydrochloride (mean value ± S.D., $n = 10$).

Parameters	Mean	Standard deviation
T_{max} (h)	21.0	3.0
C_{max} (ng/ml)	0.18	0.02
AUC_{0-t} (ng h/ml^{-1})	5.95	0.85
$t_{1/2}$ (h)	22.30	5.20

C_{max}, peak drug concentration, obtained directly from the original concentration–time data; T_{max}, time to peak drug concentration, obtained directly from the original concentration–time data; AUC_{0-t}, area under the concentration–time curve from time zero to the last sampling time 120 h, calculated using log linear trapezoidal rule; and $t_{1/2}$: terminal elimination half-life, where is the elimination rate calculated using the semi-log linear regression from the terminal phase of concentration–time curve.

4. Conclusion

In this study, a sensitive LC–MS/MS assay for plasma butenafine concentration determination was developed and validated. This method offers a rapid and reliable approach for determination of plasma butenafine concentrations, which is applicable to clinical pharmacokinetic study of butenafine following topical administration of a regular clinical dose.

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