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Short communication

High-performance liquid chromatography electrospray ionization mass spectrometry determination of tulobuterol in rabbit's plasma

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Abstract

A sensitive and specific liquid chromatography electrospray ionization mass spectrometry (LC–ESI–MS) method has been developed and validated for the identification and quantification of tulobuterol in rabbits' plasma. After the addition of clenbuterol-HCl, the internal standard (IS) and 1.0 M sodium hydroxide solution, plasma samples were extracted using a solvent mixture comprised of 5% isopropanol in *n*-hexane. The compounds were separated on a prepacked Lichrospher CN (5 μm, 150 mm × 2.0 mm) column using a mixture of methanol–water (10 mM CH_3COONH_4, pH 4.0) as mobile phase. A Shimadzu LCMS-2010A mass spectrometer connected to a Shimadzu high performance liquid chromatograph (HPLC) was used to develop and validate the method. The method has shown to be sensitive and specific by testing six different blank plasma batches. Linearity was established for the range of concentrations 0.50–40.0 ng/mL with a coefficient of determination (*r*) of 0.9998. The intra-day precision was better than 15%. The lower limit of quantification (LLOQ) was identifiable and reproducible at 0.50 ng/mL. The proposed method enables the unambiguous identification and quantification of tulobuterol for pharmacokinetic, bioavailability or bioequivalence studies.

Keywords: Tulobuterol; HPLC–ESI–MS; Rabbits' plasma; Pharmacokinetics

1. Introduction

Tulobuterol-HCl {α-[(*tert*-butylamino)methyl]-*o*-chlorobenzyl alcohol hydrochrode} (Fig. 1) is a β_2-adrenoceptor agonist having a potent and long-lasting effect [1–4].

Determination of the pharmacokinetic profile of tulobuterol is important for gaining a better understanding of its mechanism of action and for ensuring more efficient therapeutic application. Because of the low therapeutic dose of tulobuterol-HCL (1–3 mg/day for oral, 400–800 μg/day for inhalation and 2–6 mg/day for transdermal), a sensitive analytical method is needed for its determination in plasma after administration orally or transdermally.

Matsumura et al. [5–8] has developed several gas chromatographic methods coupled with electron capture detector (ECD) or mass spectrometry to determination tulobuterol and its metabolites in human serum and animal urine, the LLOQ of tulobuterol in serum were 0.5 and 1 ng/mL, respectively. Wood et al. [9] established a sensitive method for the measurement of tulobuterol in plasma and urine by capillary gas chromatography with ECD and the LLOQ was 0.2 ng/mL. But the sample preparation and extraction procedure of the methods mentioned above were time consuming. Thienpont et al. [10] reported a method for the quantification of tulobuterol in human plasma, based upon selective extraction and high resolution capillary gas chromatography–mass spectrometry, However, the LLOQ was only 170 ng/mL, and it could not have the sufficient sensitivity to properly evaluate the pharmacokinetics of tulobuterol in human or in rabbit when tulobuterol was given transdermally with lower dose.

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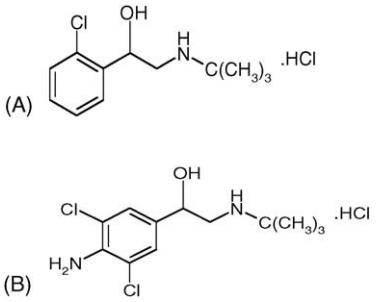

Fig. 1. Chemical structures of tulobuterol-HCl (A) and clenbuterol-HCl (B).

As an effort to reduce the time required for drugs testing in biological fluids, our laboratory is continually investigating new strategies for improving sample preparation, chromatography and mass spectrometric detection. The aim of the present study was to combine a fast high performance liquid chromatography technique with mass spectrometry in order to validate a robust and reproducible reversed-phase LC–MS method for tulobuterol determination in rabbit plasma and to increase dramatically sample throughput. Our interest was to establish a simple single step extraction technique and, at the same time, to employ the affordable quadrupole MS system. This method was to be validated to ensure the proper quantification of tulobuterol in rabbit plasma down to the concentration limit of 0.50 ng/mL. At the same time, it was expected that the method would be efficient in analyzing large number of plasma samples supporting pharmacokinetic, bioavailability or bioequivalence studies after therapeutic doses of tulobuterol.

2. Experimental

2.1. Chemicals and reagents

Tulobuterol test patch was supplied by Peking Wanquan compony (Peking, PR China); tulobuterol reference patch was purchased from Hokuriku Seiyaku Co Ltd (Japan); Methanol was chromatographic pure grade and purchased from Merck (Merck Company, Germany). Other chemicals were all of analytical grade. Deionized water was distilled before using. Other reagents were used as received.

2.2. Instrumentation and operating conditions

Liquid chromatography was performed on a Shimadzu LC-10AD HPLC system consisting of an autosampler (SIL-HTc). The HPLC was coupled to a Shimadzu LCMS-2010A single quadrupole mass spectrometer with an electrospray ionization (ESI) interface. Data acquisition and processing were accomplished using Shimadzu LCMS solution software for LCMS-2010 high-performance liquid chromatography/mass spectrometer.

Chromatographic separation was carried out at 30 °C with Lichrospher CN (5–150 mm × 2.0 mm) column. The mobile phase consisted of methanol–water (10 mM CH_3COONH_4, pH was adjusted to 4.0 by acetic acid) = 70:30 (v/v) was set at a flow rate of 0.2 mL/min. The ESI source was set at the positive ionization mode. The $[M + H]^+$, *m*/*z* 228.05 for tulobuterol and $[M + H]^+$, *m*/*z* 277.00 for clenbuterol were selected as detecting ions, respectively. The MS operating conditions were optimized as follows: drying gas 1.5 L/min, CDL temperature 250 °C, block temperature 200 °C and probe voltage: +4.5 kV.

2.3. Preparation of stock solutions

Primary stock solutions of tulobuterol-HCl for preparation of standards and quality controls (QC) were prepared from separate weighings. The primary stock solutions were prepared in methanol:water = 70:30, v/v at a concentration of 1.0 mg/mL, and were stored at 4 °C.

The internal standard stock solution was prepared by dissolving 10.0 mg of clenbuterol-HCl in 10 mL of methanol:water = 70:30, v/v producing a concentration of 1.0 mg/mL, and was stored at 4 °C.

Working solutions of tulobuterol were prepared daily in methanol:water = 70:30, v/v by appropriate dilution at 10.0, 100.0 and 1000.0 ng/mL.

2.4. Calibration curves

Calibration curves were prepared by spiking different samples of 1 mL blank plasma each with one of the abovementioned working solutions to produce the calibration curve points equivalent to 0.50, 1.0, 2.0, 4.0, 10.0, 20.0 and 40.0 ng/mL of tulobuterol-HCl. Each sample also contained 6.0 ng of the internal standard. In each run, a plasma blank sample (no IS) was also analyzed. Calibration curves were prepared by determining the best-fit of peak area ratios (peak area of analyte/peak area of IS) versus concentration, and fitted to the equation $R = bx + a$ by unweighted least-squares regression.

2.5. Preparation of quality control samples

Quality control samples were prepared at three different concentration levels, low limit, middle level and high level. QC samples were prepared daily by spiking different samples of 1 mL blank plasma each with the corresponding standard solution to produce a final concentration equivalent to 0.50, 4.0 and 40.0 ng/mL of tulobuterol-HCl and 6.0 ng of internal standard.

2.6. Extraction procedure

QC, calibration curve and clinical plasma samples were extracted employing a liquid–liquid extraction technique. To each tube containing 1 mL plasma, 6.0 ng of internal

standard, 200 μL of 1.0 M sodium hydroxide solution and 5 mL isopropanol: *n*-hexane. (5:95, v/v) were added and vortexed for 2 min. Afterwards, samples were centrifuged for 10 min at 4000 × *g*. The organic layer was evaporated under a stream of nitrogen at 40 °C. The residue was redissolved in 100 μl mobile phase. An aliquot of 10 μL was injected into the LC–MS system.

2.7. Method validation

The method validation assays were carried out according to the currently accepted US Food and Drug Administration (FDA) bioanalytical method validation guidance [11]. The following parameters were considered.

The method's specificity was tested by screening six different batches of healthy human blank plasma. Each blank sample was tested for interference using the proposed extraction procedure and chromatographic/spectroscopic conditions and compared with those obtained with an aqueous solution of the analyte at a concentration near to the LLOQ.

The matrix effect on the ionization of analytes was evaluated by comparing the peak area of analytes resolved in blank sample (the final solution of blank plasma after extraction and reconstitution) with that resolved in mobile phase. Three different concentration levels of tulobuterol (0.50, 4.0 and 40.0 ng/mL) were evaluated by analyzing six samples at each level. The blank plasma used in this study were six different batches of healthy human blank plasma. If the ratio <85% or >115%, an exogenous matrix effect was implied.

Linearity was tested for the range of concentrations 0.50–40.0 ng/mL. For the determination of linearity, standard calibration curves of at least seven points (non-zero standards) were used. In addition, a blank plasma sample were also analyzed to confirm absence of interferences, these sample was not used to construct the calibration function. The acceptance criteria for correlation coefficient was 0.998 or more, otherwise the calibration curve should be rejected. Five replicate analyses were done.

The intra-day precision and accuracy of the assay was measured by analyzing five spiked samples of tulobuterol at each QC level (0.50, 4.0 and 40.0 ng/mL). The inter-day precision and accuracy was determined over five days by analyzing 15 QC samples. The acceptance criteria for precision and accuracy deviation values should be within 15% of the actual values.

The extraction yield (or absolute recovery) was determined by comparing the tulobuterol/IS peak area ratios obtained following the outlined extraction procedure (the procedure was a little different from the outlined extraction procedure for QC, calibration curve and clinical plasma samples, that is IS was added to the organic layer after the extraction of tulobuterol) and the result compared with those obtained from samples which contained the same amount of tulobuterol in extracted plasma but not be extracted after addition of the drug. This procedure was repeated for the three different concentrations of tulobuterol added, namely 0.50, 4.0 and 40.0 ng/mL.

For sensitivity determination, the lowest standard concentration in the calibration curve was considered as the lower limit of quantification, and was to meet the following criteria: LLOQ response should be ten times the response of the blank and the LLOQ response should be identifiable, discrete and reproducible with a precision corresponding to a maximum 20% R.S.D. The 0.50 ng/mL concentration was investigated as the lower limit of quantification. Reproducibility and precision were also determined.

To evaluate stability on repeat analysis of samples, freeze-thaw stability was determined for three concentrations of tulobuterol in plasma. QC plasma samples were tested after three freeze (−20 °C) and thaw (room temperature) cycles.

3. Animals and sample collection

This was an open random, balanced parallel study in 20 rabbits. Each rabbit received in random order, single transdermal dose of 7.0 mg tulobuterol test patch (3.5 mg/3.5 cm^2 × 2) or reference patch (0.5 mg/2.5 cm^2 × 2). Blood samples(5 mL) for assay of plasma concentration of tulobuterol were collected at the time of 0, 1, 2, 4, 6, 8, 10, 12, 15, 20, 24, 30 and 36 h after transdermal administration of the medicals. Blood was taken into lithium heparin tubes, centrifuged at 3000 × *g* for 10 min to obtain plasma for assay of tulobuterol, which was then frozen at −20 °C until analyzed.

4. Results and discussion

4.1. Selection of IS

It is necessary to use an IS to get high accuracy when a mass spectrometer is used as a HPLC detector. Clenbuterol-HCl [12] was adopted in the end because of its similarity of structure (Fig. 1), retention and ionization with the analyte and the less endogenous interferences at tulobuterol $[M + H]^+$, *m*/*z* 228.05.

4.2. Sample preparation

Liquid–liquid extraction [5–9] was necessary and important because this technique can not only purify but also concentrate the sample. Ethyl acetate, methylene chloride: ethyl acetate mixture (20:80, v/v) and isopropanol: *n*-hexane (5:95, v/v) were all tested to do extraction and the isopropanol: *n*-hexane mixture (5:95, v/v) was finally adapted because of its high extraction efficiency. NaOH (1.0 M, 0.2 mL) was added to the plasma in order to accelerate the drugs' dissociation from the plasma and reduce interference since most endogenous are of acidic nature.

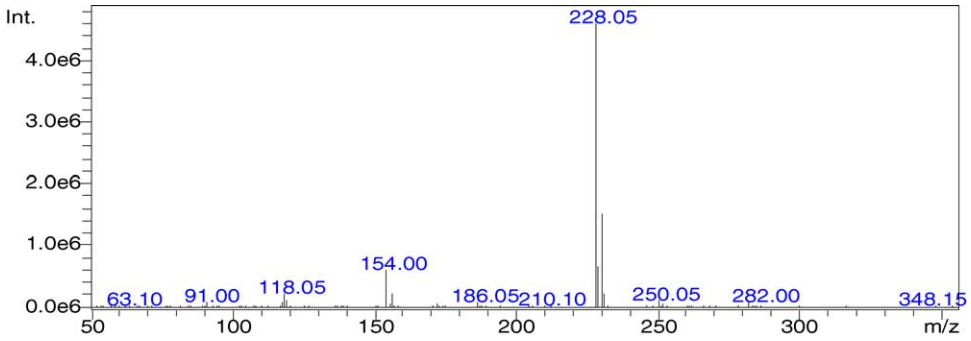

Fig. 2. Positive ion electrospray mass scan spectrum of tulobuterol.

4.3. Separation and specificity

Positive ion electrospray mass scan spectrum of tulobuterol and IS was shown in Figs. 2 and 3, respectively. The major ions observed were $[M + H]^+$ isotope peaks at *m/z* 228.05, 230.05 for tulobuterol and $[M + H]^+$ isotope peaks at *m/z* 277.00, 279.05 for clenbuterol. The ions of $[M + H]^+$, *m/z* 228.05 for tulobuterol and $[M + H]^+$, *m/z* 277.00 for clenbuterol were finally selected for the SIM (+) mode for their stability and high intensity.

The SIM (+) chromatograms extracted from supplemented plasma are depicted in Fig. 4(B). As shown, the retention times of tulobuterol and IS were 4.7 and 5.2 min, respectively.

The total HPLC–MS analysis time was 6.0 min per sample. No interferences of the analyte were observed because of the high selectivity of the SIM technique. No ion suppression effects were observed under the developed sample preparation and chromatographic conditions. Fig. 4(A) shows a HPLC chromatogram for a blank plasma sample indicating no endogenous peaks at the retention times of tulobuterol or internal standard (clenbuterol). The SIM (+) chromatograms obtained from an extracted plasma sample of a rabbit that participated in a bioequivalence study conducted on 20 rabbits are depicted in Fig. 5.

The purpose of these investigations was to develop a specific and sensitive assay for the determination of tulobuterol used as a β_2-adrenoceptor. HPLC–ESI–MS has several advantages for the analysis of tulobuterol. The combination of HPLC (under the isocratic conditions described) with ESI–MS leads to short retention time and yields both high selectivity and sensitivity. ESI is a 'gentle' ionization technique that produces high mass-to-charge $[M + H]^+$ precursor ions with minimal fragmentation of the analyte.

4.4. Method validation

The method exhibited a good linear response for the range of concentrations from 0.50 to 40.0 ng/mL. Results of five representative calibration curves for tulobuterol HPLC/MS determination are given in Table 1. The table also shows the back-calculated concentrations for each point and the mean recovery. The function of mean calibration curve is $R = 0.3893x + 0.0382$ with a coefficient of determination (r) of 0.9998.

Data for the intra-day precision and the inter-day precision of the method for tulobuterol as determined from the QC samples run, at the concentrations of 0.50, 4.0 and 40.0 ng/mL, are presented in Table 2.

The lower limit of quantification for tulobuterol was proved to be 0.50 ng/mL and the lower limit of detection for tulobuterol was 0.10 ng/mL. Fig. 6 shows the chromatogram of an extracted sample that contained 0.10 ng/mL of tulobuterol (LLOD).

The extraction recovery determined for tulobuterol was shown to be consistent, precise and reproducible. The mean recoveries of the low, middle and high QC levels were

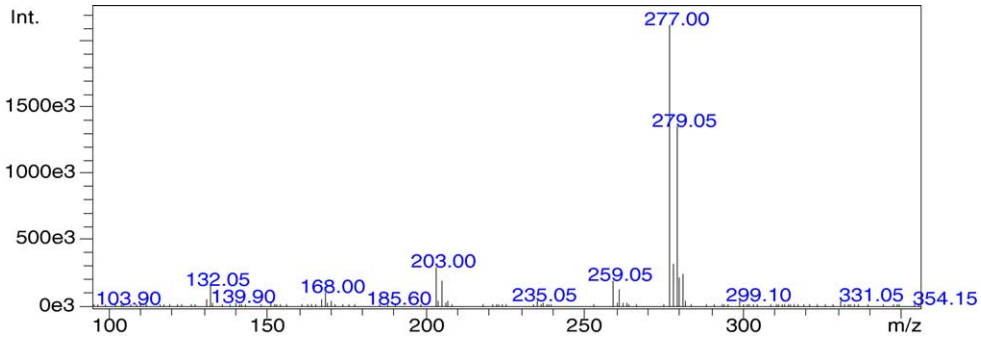

Fig. 3. Positive ion electrospray mass scan spectrum of clenbuterol.

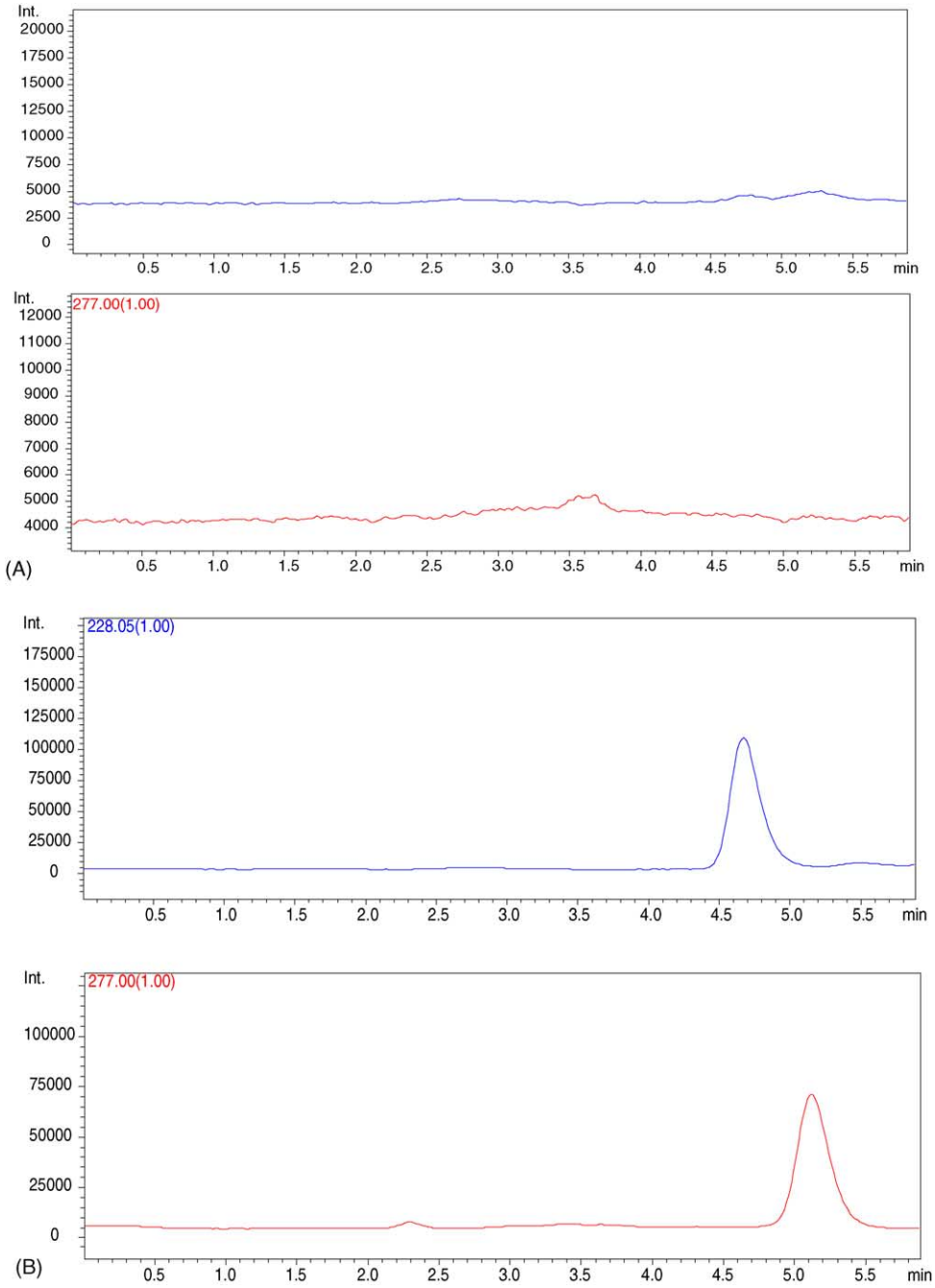

Fig. 4. The SIM (+) chromatograms of (A) blank plasma sample and (B) blank plasma spiked with tulobuterol and IS. The retention times of tulobuterol and IS were 4.7 and 5.2 min, respectively.

94.30, 94.25 and 92.40%, respectively whereas the precision (R.S.D.) were 7.73, 4.56 and 5.00%, respectively.

4.5. Stability

The results of freeze–thaw stability indicated that the analyte is stable in plasma for three freeze–thaw cycles, when stored at −20 °C and thawed to room temperature. Long-term stability indicates that storage of tulobuterol plasma samples at 20 °C is adequate when stored for 9 days and no stability-related problems would be expected during routine analysis for the pharmacokinetic, bioavailability or bioequivalence studies.

4.6. Results of pharmacokinetic study

The method was applied to analyze plasma samples obtained after the transdermanal administration of 7.0 mg of

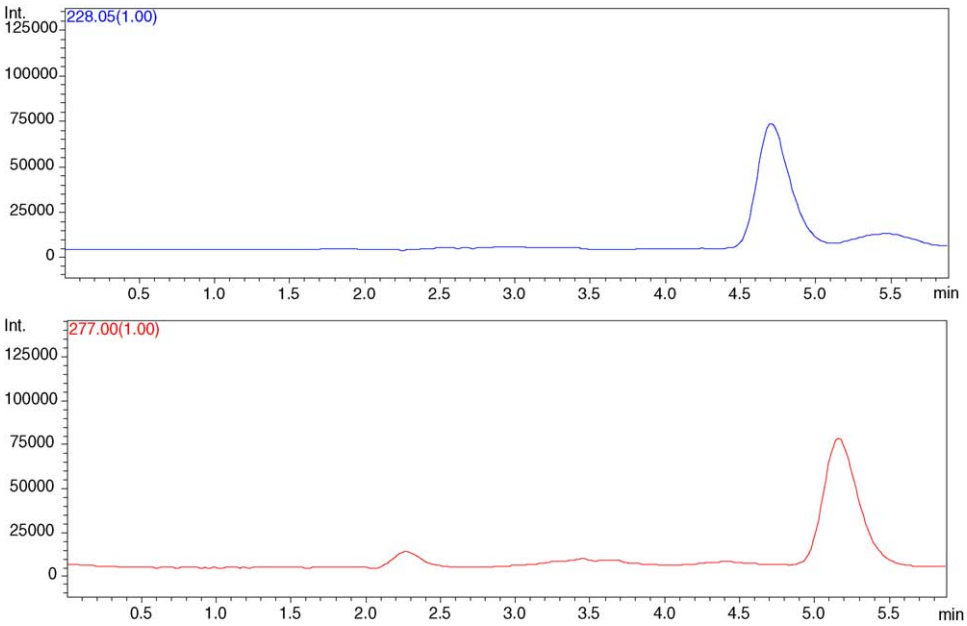

Fig. 5. The SIM (+) chromatogram for plasma sample of a rabbit. The retention times of tulobuterol and IS were 4.7 and 5.2 min, respectively.

Table 1
Results of five representative calibration curves for tulobuterol LC–MS determination

Calibration curves	Concentration of standards (ng/mL)						
	0.5	1.0	2.0	4.0	10.0	20.0	40.0
First	0.49497	1.05173	2.37359	4.28734	9.47736	19.42526	40.54395
Second	0.42204	0.98552	2.17301	4.45506	8.90964	18.89404	39.50210
Third	0.47455	1.03682	1.84423	4.45506	10.41344	20.19675	39.73211
Fourth	0.52890	1.08286	1.84440	4.54164	10.13774	20.98310	42.79930
Fifth	0.48491	1.25510	1.90335	3.88133	10.01341	18.20299	38.66267
Mean	0.48107	1.08241	2.02772	4.32408	9.79032	19.54043	40.24802
R.S.D.%	8.07	9.49	11.66	6.11	6.11	5.57	3.92
Mean recovery%	96.21	108.24	101.39	108.10	97.90	97.70	100.62

Table 2
Precision of the method with determination of tulobuterol ($n = 5$)

Added concentration (ng/mL)	Intra-day			Inter-day		
	Mean (ng/mL)	R.S.D.%	Accuracy%	Mean (ng/mL)	R.S.D.%	Accuracy%
0.5	0.5105	10.82	102.11	0.4844	13.36	96.21
4.0	4.2707	9.04	106.77	4.3256	6.11	108.10
40.0	41.5462	5.73	103.87	40.2496	3.92	100.62

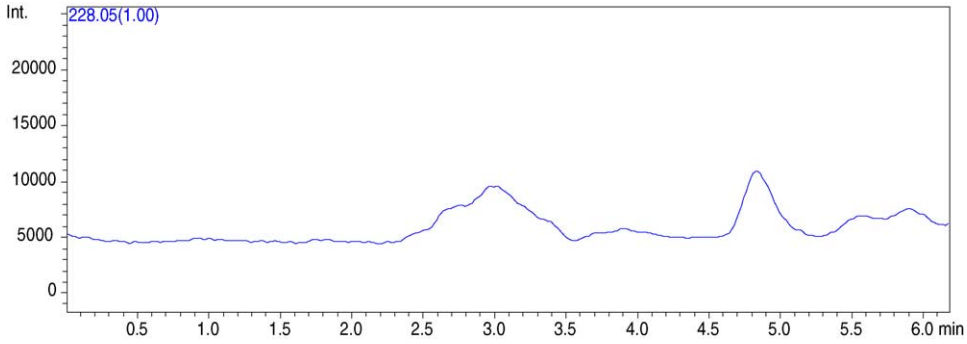

Fig. 6. LLOD (0.1 ng/mL) of tulobuterol, retention time was 4.7 min.

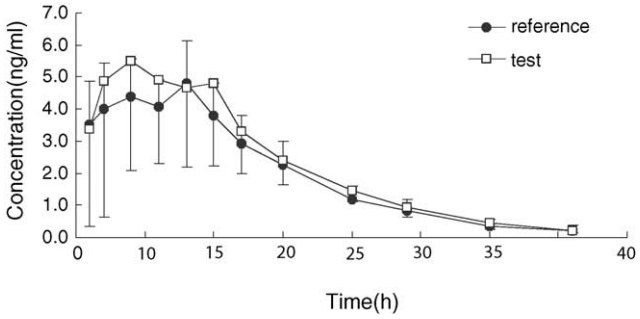

Fig. 7. Mean drug plasma concentration-time curve of tulobuterol in 20 rabbits after transdermal administration.

Table 3
Pharmacokinetic parameters of tulobuterol in rabbits after transdermal administration

Parameters	Test patch	Reference patch
T_{max}/h	6.4 ± 3.6	5.5 ± 3.2
$T_{1/2}$ K/h	6.08 ± 0.81	6.36 ± 2.30
$MRT_{0\rightarrow\infty}$/h	12.11 ± 1.88	11.82 ± 1.78
C_{max}/ng mL^{-1}	6.34 ± 4.08	5.53 ± 1.17
$Auc_{0\rightarrow 36}$/ng h mL^{-1}	83.16 ± 38.20	72.45 ± 12.88
$Auc_{0\rightarrow\infty}$/ng h mL^{-1}	85.18 ± 38.50	74.89 ± 13.91

tulobuterol test patch (3.5 mg/3.5 cm^2 × 2) to 20 rabbits participating in bioequivalence studies. The procedure developed was sensitive enough to assure the quantitative analysis of tulobuterol in plasma with acceptable accuracy over a period of 36 h after a single transdermal administration. The mean plasma concentration-time profiles of 20 rabbits are represented in Fig. 7. Pharmacokinetic parameters of the test patch and the reference patch are listed in Table 3. The test patch was found to be bioequivalent to the reference one.

5. Conclusion

The proposed method of analysis provided a sensitive and specific assay for tulobuterol determination in rabbit plasma. Simple liquid–liquid extraction procedure and short run time can provide a short analysis time that is important for large sample batches. It was shown that this method is suitable for the analysis of tulobuterol in plasma samples collected for pharmacokinetic, bioavailability or bioequivalence studies.

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