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Determination of isotretinoin in human plasma by high performance liquid chromatography–electrospray ionization mass spectrometry

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ABSTRACT

A rapid, sensitive and specific high performance liquid chromatography–electrospray ionization mass spectrometry (LC–ESI-MS) method for the quantification of 13-cis-retinoic acid (isotretinoin) in human plasma has been developed. Acitretin was employed as the internal standard (IS). The analytes were chromatographically separated on a Shimadzu Shim-pack VP-ODS C18 column (150 mm × 2.0 mm I.D.) with a mobile phase consisting of acetonitrile and water (90:10, v/v). Detection was performed on a single quadrupole mass spectrometer using an electrospray ionization interface with the selected-ion monitoring (SIM) mode. The method showed excellent linearity (r = 0.9989) over the concentration range of 10–1500 ng/mL with good accuracy and precision. The intra- and inter-batch precisions were within 10% relative standard deviation. The recoveries were more than 80%. The validated method was successfully applied to a preliminary bioequivalence study of isotretinoin in 20 Chinese healthy male volunteers.

1. Introduction

Oral isotretinoin [13-cis-RA, (2Z,4E,6E,8E)-3,7-dimethyl-9-(2,6, 6-trimethyl-1-cyclohexenyl)nona-2, 4, 6, 8-tetraenoic acid, MW = 300.44] revolutionized the treatment of sever nodulo-cystic acne when it was introduced in 1979 [1]. A single course of therapy at recommended doses from 0.5 to 2 mg/kg in 2 doses per day for 15–20 weeks has been shown to result in complete and prolonged remission of the disease in most patients. Some publications report that oral isotretinoin significantly reduces the total number of resistant bacteria on the skin of patients with acne [2,3]. Today isotretinoin has been widely used for the treatment of certain disorders of keratinization [4,5] as an effective antiacne drug. There is also interest in the prophylactic and therapeutic effects of 13-cis-RA in in vitro and in vivo carcinogenesis studies [6], pigmentary disorders [7] and photoaging [8].

Various chromatographic methods have been reported for the quantitative determination of isotretinoin and its metabolite in biological samples, such as HPLC with ultraviolet (UV) detection [9–16], mass spectrometry [17–19] or tandem mass spectrometry [20]. However, most of the published methods often suffer from disadvantages, including poor sensitivity, long running time and cumbersome and complex analytical procedures. The use of HPLC/UV with column switching technique (on-line solid-phase extraction) appeared to be the most promising. But the complicated processing technique and the requirement for a large amount of biological sample may limit its wide application [21–23].

In this paper, we describes a simple, selective and highly sensitive method by using high performance liquid chromatography coupled with electrospray ionization single quadrupole mass spectrometry for the determination of isotretinoin in human plasma. The method is validated over the concentration range of 10–1500 ng/mL for isotretinoin. And it has been successfully applied to a preliminary bioequivalence study of isotretinoin in 20 healthy male Chinese volunteers.

2. Experimental

2.1. Laboratory precautions

As there was long electron rich conjugated polyene chain in its structure, isotretinoin was easily influenced by many factors, such as light with wavelength less than 500 nm, oxygen, trace metal, strong acid, relative high heat, etc. The preparetion of reference compounds solution and biological samples was performed in a darkened room illuminated with red light, whenever possible amber containers were used.

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2.2. Chemicals

Isotretinoin reference standard (100.0% purity) were supplied and identified by ChongqingHuaPont Pharmaceutical Co., Ltd. (Chongqing, PR China). Acitretin reference standard (internal standard, 100.2% purity) was supplied and identified by HanJiang pharmaceutical factory (Shanxi, PR China). Isotretinoin test capsules were supplied and identified by ChongqingHuaPont Pharmaceutical Co., Ltd. (Chongqing, PR China), isotretinoin reference capsules were supplied and identified by Shanghai Sine Pharmaceutical (Group) Co., Ltd. (Shanghai, PR China). Acetonitrile (Darmstadt, Germany), hexane (Honeywell International Company, USA) and isopropanol (Jiangsu hanbon science and technology Co., Ltd., Nanjing, PR China) were of chromatographic grade. N,N-dimethylformamide (Nanjing Chemical Reagent Co., Ltd., Nanjing, PR China) was of analytical grade. The mobile phase was filtrated with a 0.22-μm film before use. Water was purified by redistillation before use.

2.3. LC–MS instrument and conditions

Liquid chromatographic separation and mass spectrometric detection were performed using a Shimadzu LC/MS system consisting of a Shimadzu LC-20AD binary pump, a Shimadzu SIL-20AC auto sampler, a Shimadzu CTO-20A column oven and a Shimadzu 2010EV single quadrupole mass spectrometer with ESI interface. Liquid chromatographic separation was achieved on a Shimadzu Shim-pack VP-ODS C18 column (150 mm × 2.0 mm I.D.) column which was maintained at 40 °C. The mobile phase consisting of acetonitrile and water (90:10, v/v) was pumped at an isocratic flow rate of 0.2 mL/min. The total running time was 7.0 min for each injection. The mass spectrometer was operating in negative ion and SIM (selective ion monitoring) mode. The $[M–H]^-$, m/z 299.05 for isotretinoin and $[M–H]^-$, m/z 325.00 for acitretin were selected as detecting ions, respectively. The MS operating conditions were optimized as follows: nebulizer gas rate 1.5 L/min, CDL temperature 250 °C, block temperature 200 °C, probe voltage −4.0 kV. Mass spectra were obtained with a scan speed of 500 amu/s in scan mode and an event time of 1 s in SIM mode. The peak width setting (FWHM) was 0.6 μm. The quantification was performed via peak-area ratio. Data acquisition and processing were accomplished using Shimadzu LCMS solution Version 3.30 for LCMS-2010EV system.

2.4. Preparation of stock solutions

Stock solution of isotretinoin was prepared in methanol at the concentration of 500 μg/mL. The stock solution of acitretin (IS) was prepared in methanol with N,N-dimethylformamide as cosolvent at the concentration of 500 μg/mL. Working solutions of isotretinoin were prepared daily in methanol by appropriate dilution to the final concentration of 100, 200, 400 ng/mL 1, 2, 4, 10, 20, 24 and 30 μg/mL. The stock solution of acitretin was further diluted with methanol to prepare the working internal standard solution containing 10 μg/mL of acitretin.

2.5. Sample preparation

Liquid–liquid extraction was chosen for the sample preparation. A 0.5 mL aliquot of the collected plasma sample from a human volunteer was pipetted into a 10 mL glass centrifuge tube. The working internal standard solution (25 μL × 10 μg/mL) and 4.0 ml hexane and isopropanol (95:5, v/v) were added. The mixture was vortexed for 3 min. After centrifugation at 1330 × g for 10 min at room temperature, the upper organic layer was transferred to another 10 mL centrifuge tube and evaporated to dryness under a gentle stream of nitrogen gas in a water bath at 37 °C. The residues were then redissolved in 100 μL mobile phase under vortex and centrifuged at 20,627 × g for 8.0 minutes. 10 μL aliquots of the supernatant were injected into the LC–MS system.

2.6. Standard curves and quality control samples

Human blank plasma samples for the development, validation and control of the method were obtained from healthy, drug-free volunteer blood donors at Jiangsu Provincial Blood Center (Nanjing, PR China). Calibration curves were prepared by spiking blank plasma with appropriate volume of one of the above-mentioned working solutions to produce the standard curve points equivalent to 10 (25 μL × 200 ng/mL), 50 (25 μL × 1 μg/mL), 100 (25 μL × 2 μg/mL), 200 (25 μL × 4 μg/mL), 500 (25 μL × 10 μg/mL), 1000 (25 μL × 20 μg/mL), 1500 (25 μL × 30 μg/mL) ng/mL of isotretinoin. Each sample also contained 500 (25 μL × 10 μg/mL) ng/mL of the internal standard. The following assay procedures were the same as that described above. Blank plasma sample (without IS) was also analyzed. Quality control (QC) samples were prepared by spiking blank plasma with appropriate volume of one of the working solution mentioned above to produce a final concentration equivalent to 20 ng/mL (25 μL × 400 ng/mL, low level), 500 ng/mL (25 μL × 10 μg/mL, middle level) and 1200 ng/mL (25 μL × 24 μg/mL, high level) of isotretinoin with 500 ng/mL of internal standard each. The following procedures were the same as that described above.

2.7. Biomedical method validation

The method validation assays were carried out according to the United States Food and Drug Administration (FDA) bioanalytical method validation guidance [24]. Calibration curves were prepared by determining the best-fit of peak area ratios (peak area of analyte/peak area of internal standard) versus concentration, and fitted to the equation $R = bC + a$ by weighted least-sqaure linearity regression. Blank samples of healthy human plasma used for testing specificity of the method were obtained from six different sources supplied by Jiangsu Provincial Blood Center (Nanjing, PR China). The visible interferences were tested with blank plasma samples and plasma samples with isotretinoin concentrations close to the lower limit of quantification (LLOQ).

The potential matrix effect on the ionization of the analyte was evaluated by comparing the peak area of the analyte dissolved in the supernatant of the processed blank plasma to that of standard solutions at the same concentration. Three different concentration levels of isotretinoin (20, 500 and 1200 ng/mL) were evaluated by analyzing five samples at each concentration level. The matrix effect of the internal standard (500 ng/mL in plasma) was evaluated using the same method.

The lower limit of detection (LLOD) and the lower limit of quantification (LLOQ) were determined as the concentrations with a signal-to-noise ratio of 3 and 10, respectively. Each concentration standard should meet the following acceptable criteria: no more than 20% deviation at LLOQ and no more than 15% deviation for the standards above LLOQ. The intra-batch precision and accuracy was determined by repeated analysis of five spiked samples of isotretinoin at each QC level (20, 500 and 1200 ng/mL) on one day ($n = 5$).

Inter-batch precision and accuracy was determined by repeated analysis on three consecutive days ($n = 5$ series per day). The concentration of each sample was calculated using standard curve prepared and analyzed on the same day. The precision was defined as the RSD (relative standard deviation, %) and the accuracy was expressed as a percentage of the measured concentration over the theoretical concentration. The acceptance criteria for intra- and

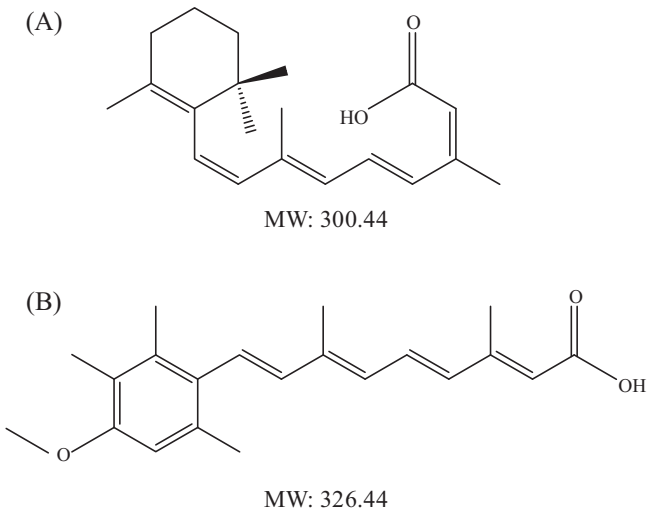

Fig. 1. Chemical structure of isotretinoin (A) and acitretin (B).

inter-batch precisions were within 15% and accuracy did not exceed 15%.

The absolute extraction recovery of isotretinoin was determined by comparing the isotretinoin/I.S. peak area ratios obtained from extracted plasma samples with those from post-extracted blank plasma samples spiked at corresponding concentrations. This procedure was repeated ($n = 5$) at each QC level (20, 500 and 1200 ng/mL).

Freeze and thaw stability: Two concentration levels of QC plasma samples (20 and 1200 ng/mL) were tested after three freeze (−20 °C)-thaw (room temperature) cycles. Short-term temperature stability: Two concentration levels of QC plasma samples (20 and 1200 ng/mL) were kept at room temperature for a period that exceeded the routine preparation time of samples (around 8 h).

Long-term stability: Two concentration levels of QC plasma samples (20 and 1200 ng/mL) kept at low temperature (−20 °C) were studied for a period of 20 days.

Auto sampler stability: The auto sampler stability was conducted by reanalyzing extracted QC samples (20 and 1200 ng/mL) kept under the auto sampler conditions (4 °C) for 24 h.

Stock solution stability: The stability of isotretinoin and internal standard working solutions were evaluated at room temperature for 6 h.

3. Results and discussion

3.1. Selection of IS

Acitretin was selected as IS because of its retention, ionization and extraction efficiency were found to be appropriate among the compounds we tried. The structures of isotretinoin and acitretin are shown in Fig. 1.

3.2. Sample preparation

N-hexane-isopropanol (95:5, v/v), ethyl acetate and ethyl ether were all attempted and n-hexane-isopropanol (95:5, v/v) was finally adopted because of its high extraction efficiency and less interference.

3.3. LC–MS optimization

Both the positive and negative modes were investigated and the response of negative ions was stronger than positive ions,

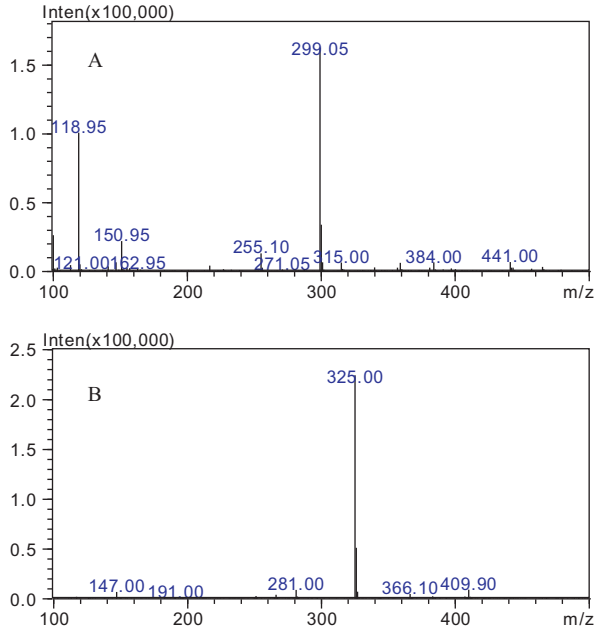

Fig. 2. Negative ion electrospray mass scan spectra of isotretinoin (A) and acitretin (B).

which indicate that the negative mode was much more sensitive. Mobile phases such as acetonitrile–water, methanol–water, acetonitrile–formic acid solution, methanol–formic acid solution, acetonitrile-aqueous ammonium acetate solution (10 mM) and methanol-aqueous ammonium acetate solution (10 mM) with different ratios were also investigated for optimization. Finally, acetonitrile–water (90:10, v/v) was chosen as the mobile phase because of the symmetric peak shape and appropriate retention data for isotretinoin and IS. Negative electrospray ionization mass scan spectra of isotretinoin and IS are shown in Fig. 2, respectively. According to the mass scan spectra, The $[M–H]^-$, m/z 299.05 for isotretinoin and $[M–H]^-$, m/z 325.00 for acitretin were selected for monitoring.

The SIM (−) chromatograms extracted from supplemented plasma are depicted in Fig. 4. As shown, the retention times of isotretinoin and the IS were 5.4 and 3.9 min, respectively. Good separation of analytes and short retention times were obtained by using an elution system consisting of acetonitrile and water (90:10, v/v) as the mobile phase. The total HPLC/MS analysis time was 7.0 min per sample.

3.4. Assay specificity and matrix effect

No analyte-interfering peaks were observed due to the selectivity of SIM. Drug-free plasma was extracted with n-hexane-isopropanol (95:5, v/v) and the results were recorded. Fig. 3 shows the representative HPLC chromatogram for a drug-free plasma sample, indicating that no endogenous peaks are present at the retention time (t_R) of isotretinoin or of the IS. Five replicates for each of three concentrations (20, 500 and 1200 ng/mL) were prepared from different batches of plasma. All the ratios of the peak area of the analytes dissolved in the supernatant of the processed blank plasma compared with that of standard solutions at the same concentrations were respectively in the range 78.5–81.4% for isotretinoin and 61.8–71.5% for IS. The results showed that the co-eluting endogenous substances had ion suppression effect on the ionization of isotretinoin and IS. While ion suppression effect was relatively high, it proved to be reproducible. And accuracy and precision of the method was acceptable. Furthermore, it did not affect

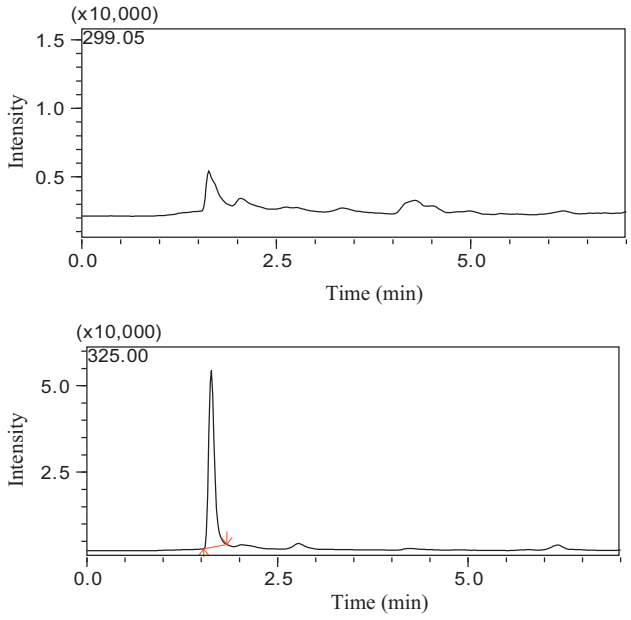

Fig. 3. The SIM (-) chromatograms of blank plasma sample.

the desired sensitivity of the assay with a LLOQ of 10 ng/mL demonstrating that the method was fit for purpose. As all the samples came from the same body fluid and had the same background, the existence of ion suppression effect would not affect the results of the specificity, sensitivity and precision of the method, also would not have effect on the results of the bioequivalence study.

3.5. Linearity of calibration curves and specificity

The method exhibited good linear response over the selected concentration range by linear regression analysis. Standard curves were constructed on 5 different days. The mean standard curve was typically described by weighted ($1/C^2$) linear regression equation: $R = 0.0032 \times C + 0.0015$, $r = 0.9989$, where R corresponds to the peak area ratio of isotretinoin to the I.S. and C refers to the concentration of isotretinoin added to plasma over a concentration range of 10–1500 ng/mL. Linear dependency between R and C was confirmed by regression analysis with SPSS15.0 ($P < 0.001$). Results of five representative standard curves for isotretinoin LC/MS determination are given in Table 1.

The lower limit of quantification for isotretinoin was proved to be 10 ng/mL (LLOQ) and the lower limit of detection (LLOD) was 5 ng/mL. Fig. 4 shows the chromatogram of an extracted sample that contained 10 ng/mL (LLOQ) of isotretinoin.

3.6. Precision and accuracy

Data for intra-batch and inter-batch precision and accuracy of the method for isotretinoin are presented in Table 2. The RSD values for intra-batch precision and accuracy were in the range 3.94–9.56% and 98.69–103.60%, respectively. Whereas the corresponding inter-batch values were 5.77–8.15% and 98.05–101.85%, respectively. The results revealed good precision and accuracy.

3.7. Extraction recovery

The extraction recovery determined for isotretinoin was shown to be consistent, precise and reproducible. The mean recoveries of the three concentration levels (20, 500 and 1200 ng/mL) were 88.36%, 85.72% and 81.39%, respectively whereas the relative standard deviations were 9.22%, 9.11% and 5.87%, respectively. Table 3 summarizes the freeze and thaw stability, short-term stability, long-term stability and auto sampler stability data of isotretinoin. All the results showed the reliable stability behavior during these tests and there were no stability-related problems during the routine analysis of samples for the bioequivalence study. The stability of working solutions was tested at room temperature for 6 h. Based on the results obtained, these working solutions were stable within 6 h.

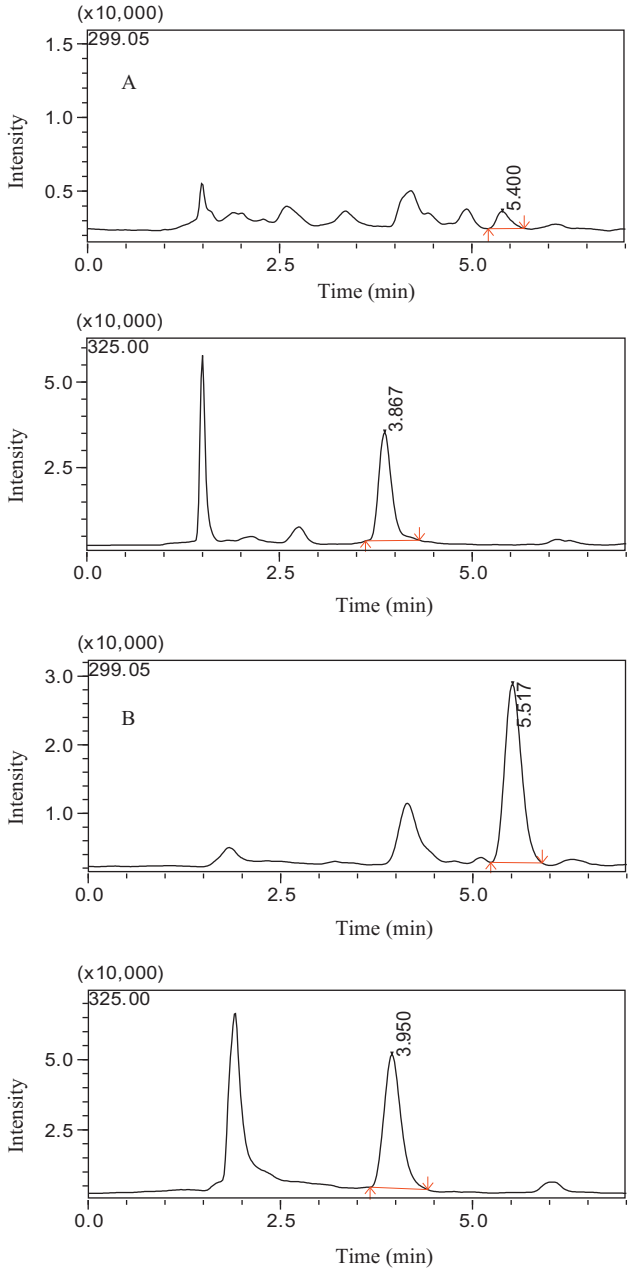

Fig. 4. The SIM (-) chromatograms of (A) LLOQ (10 ng/mL) and (B) plasma sample of a healthy volunteer. The retention times of isotretinoin (184.65 ng/mL) and the IS (500 ng/mL) were 5.5 min and 3.9 min, respectively.

3.8. Application

The validated method was successfully applied to a bioequivalence study of two formulations of isotretinoin. Twenty healthy male subjects (age 23.8 ± 3.23 years, height 173.5 ± 5.65 cm, weight 63.7 ± 5.88 kg) were enrolled in the study after the assessment

Table 1
Results of five calibration curves for isotretinoin determining in human plasma.

Added conc. (ng/mL)	Assay	10	50	100	200	500	1000	1500
Back-calculated	1	10.36	45.82	99.40	209.41	503.70	979.05	1658.27
conc. (ng/mL)	2	9.92	46.99	111.94	182.43	480.67	950.59	1546.30
	3	9.89	46.33	106.21	206.45	495.42	956.75	1390.87
	4	10.08	47.46	108.49	195.59	474.73	952.64	1617.26
	5	11.03	48.59	98.97	205.35	454.95	946.07	1585.74
Mean (ng/mL)		10.26	47.04	105.00	199.85	481.90	957.02	1559.69
SD (ng/mL)		0.47	1.07	5.69	11.03	18.96	12.90	102.94
RSD (%)		4.60	2.27	5.42	5.52	3.93	1.35	6.60
Accuracy (%)		102.57	94.07	105.00	99.92	96.38	95.70	103.98

Table 2
The precision and accuracy of the method for the determination of isotretinoin in human plasma.

Added conc. (ng/mL)	Intra-batch ($n = 5$)			Inter-batch ($n = 15$)		
	Detected conc. (mean ± SD) (ng/mL)	Accuracy (%)	Precision (%)	Detected conc. (mean ± SD) (ng/mL)	Accuracy (%)	Precision (%)
20	19.7 ± 1.9	98.7	9.6	20.4 ± 1.7	101.9	8.2
500	507.4 ± 29.5	101.5	5.8	490.2 ± 37.4	98.1	7.6
1200	1243.2 ± 48.9	103.6	3.9	1219.2 ± 70.3	101.6	5.8

Table 3
Data showing stability of isotretinoin in human plasma at different QC levels ($n = 3$).

	Accuracy (mean ± SD, %)	
	20	1200
Short-term stability (8 h, room temperature)	103.4 ± 11.7	105.4 ± 2.7
Freeze and thaw stability (3 cycles, −20 °C–room temperature)	112.9 ± 5.1	108.6 ± 6.8
Long-term stability (20 days, −20 °C)	108.8 ± 2.5	94.3 ± 2.9
Auto sampler stability (24 h, 4 °C)	113.0 ± 1.6	100.3 ± 5.6

of medical history, physical examination, electrocardiogram and standard laboratory test results (blood cell count, biochemical profile and urinalysis). The clinical protocol was approved by Institute of Dermatology, Chinese Academy of Medical Sciences and Peking Union Medical College, and all participants provided written, informed consent.

A randomized, two-period, single-dose crossover protocol with a 1-week washout interval between doses was adopted. After fasting over-night, 20 volunteers were given single dose of 40 mg isotretinoin reference capsules (*R*) or test capsules (*T*), respectively. Venous blood samples (3 mL) were collected immediately before dose and at 0.5, 1, 1.5, 2, 2.5, 3, 4, 6, 8, 12, 24, 36 and 60 h post-dosing. The plasma was separated by centrifugation at 1600 × *g* for 4 min within 20 min of blood collection, and was stored at −20 °C until analyzed.

The pharmacokinetic parameters were calculated by Bioavailability Program Package (BAPP 2.2) software. Bioequivalence of two formulations was assessed by means of analysis of variance (ANOVA) for crossover design and calculating 90% confidence interval (CI) of the ratio of test/reference using log-transformed data. The formulations were considered bioequivalent when the difference between two compared parameters was found statistically insignificant ($p > 0.05$). Furthermore, as a standard requirement, the ratio of averages of log-transformed data should be within 80–125% for AUC_{0-60}, $AUC_{0-\infty}$ and C_{max}.

A representative chromatogram of a plasma sample obtained at 4 h from a subject who received a single oral dose is shown in Fig. 4. The mean plasma concentration–time curves of 20 volunteers after oral administration of isotretinoin are showed in Fig. 5. Kinetic parameters of the test capsules and the reference capsules are listed in Table 4. The 90% CI of the individual ratios (each test formulation/reference formulation) for AUC_{0-60}, $AUC_{0-\infty}$ and C_{max} were 96.0–103.4%, 93.6–103.2% and 89.9–98.9%, respectively. The test capsule was found to be bioequivalent to the reference one.

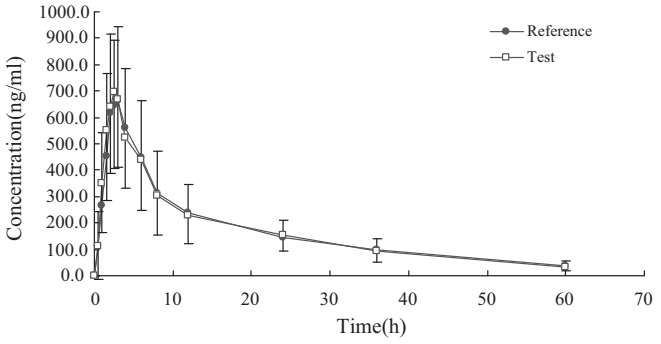

Fig. 5. Mean drug plasma concentration–time curve of isotretinoin from 20 volunteers after oral administration.

Table 4
Pharmacokinetic parameters of 20 healthy volunteers after oral administration of isotretinoin.

Parameters	Reference	Test
$T_{1/2}$ (h)	17.9 ± 4.7	17.0 ± 3.8
T_{max} (h)	2.8 ± 1.2	2.8 ± 1.0
C_{max} (ng/ml)	846.7 ± 220.1	808.1 ± 248.2
AUC_{0-60} (ng h/ml)	10059.8 ± 3116.9	9993.6 ± 2807.1
$AUC_{0-\infty}$ (ng h/ml))	11041.5 ± 3481.7	10807.6 ± 2964.0
MRT (h)	23.3 ± 6.3	22.1 ± 4.3

4. Conclusions

This paper first presented a rapid, specific and sensitive LC–ESI-MS method for determination of isotretinoin in human plasma with a total cycle time of 7.0 min for each injection. The method exhibited acceptable precision and adequate sensitivity for the quantification of isotretinoin in human plasma samples resulting from the pharmacokinetic, bioavailability or bioequivalence stud-

ies of isotretinoin. And it has been successfully applied to quantify the concentration–time profile of isotretinoin in a bioequivalence study in healthy Chinese male volunteers.

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