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Determination of benflumetol in human plasma by reversed-phase high-performance liquid chromatography with ultraviolet detection

Mei-Yi Zeng[a,*], Zhi-Liang Lu[a], Song-Cheng Yang[b], Min Zhang[a], Jie Liao[b], Shu-Ling Liu[a], Xi-He Teng[a]

[a]*Institute of Microbiology and Epidemiology, 20 Dongdaijie, Fengtai, Beijing 100071, China*
[b]*National Center of Biomedical Analysis, 27 Taiping Road, Beijing 100850, China*

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Abstract

A reversed-phase high-performance liquid chromatographic method for the determination of benflumetol in human plasma is described. Benflumetol in plasma samples was extracted with a glacial acetic acid–ethyl acetate (1:100, v/v) mixture at pH 4.0. Chromatography was performed on a Spherisorb C_{18} column using a methanol–water–glacial acetic acid–diethyl amine (93:6:1:0.03, v/v) mixture as the mobile phase and UV–VIS detection at 335 nm. The identity and purity of the benflumetol peak were carefully examined, and the internal standard method was applied for its quantitation. The absolute recovery of benflumetol in spiked plasma samples was 92.91% over the concentration range 5–4000 ng/ml. The recovery of internal standard "8212" at a concentration of 300 ng/ml in spiked plasma was 84.85%. The detection limit of benflumetol was 11.8 ng/ml. Plasma concentration–time profiles in healthy volunteer adults were measured after a single-dose oral administration of 500 mg of benflumetol. The assay procedures were within the quality control limits.

Keywords: Benflumetol

1. Introduction

Malaria is still prevalent in many countries. The most disturbing fact is that drug-resistance of malaria to antimalarials used currently is spreading rapidly, leading to a severe situation in malaria treatment globally. The search for drugs with novel types of chemical structures, which should be directed to different types of malaria and safe for use, has become an urgent subject of study.

Benflumetol is a new antimalarial drug developed in the Institute of Microbiology and Epidemiology, Beijing, China. It has been proved to be highly effective for the treatment of various types of malaria, including multi-drug-resistant *falciparum* malaria, and provides a high cure rate [1]. The objective of this work is to develop a specific and sensitive high-performance liquid chromatographic (HPLC) method for the determination of benflumetol in human plasma and to apply the method to pharmacokinetic studies in clinical trials.

Normally, synthetic benflumetol is a racemate of two stereo-enantiomers. However, there is no significant difference in antimalarial activity between the two enantiomers, and the toxicity of the racemate of benflumetol was very low [1]. Based on these results,

*Corresponding author.

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no chiral separation was attempted in this work and benflumetol was determined as a single compound.

2. Experimental

2.1. Chemicals

The reference standard benflumetol (I) and the internal standard "8212" (II), shown in Fig. 1, were provided by the Department of Medicinal Chemistry of the Institute of Microbiology and Epidemiology. All other chemicals used were purchased from the Beijing Chemical Reagents Factory (Beijing, China) and were of analytical grade, except methanol, which was of guarantee grade.

2.2. Instrumentation

The determination of I in human plasma was performed on an HPLC system equipped with a M-45 solvent delivery system (Waters, USA), a SIL-1A injector (Shimadzu, Japan), a 150 × 4.6 mm I.D. stainless steel column packed with Spherisorb C_{18} (5 μm), a SPD-2AS UV spectrophotometric detector (Shimadzu, Japan) and an A-4500 microprocessor connected to a COMX PL-80 recorder (Beijing Institute of Future Scientific Technology, China). A guard cartridge packed with C_{18} Si_2O was used. Identification and purity examination of the I peak were carried out on a 410 Bio LC Model HPLC system provided with a 235 C diode array detector (Perkin-Elmer, USA). The separation column used was a 150 × 4.6 mm I.D. stainless steel column packed with Waters Pico Tag C_{18} (5 μm).

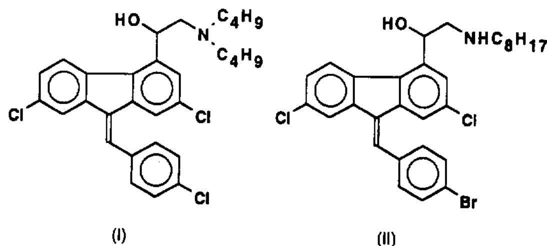

Fig. 1. Chemical structures of benflumetol (I) and "8212" (II).

2.3. Procedure

2.3.1. Preparation of standard solutions of I

A stock solution containing 1 mg/ml of I was prepared in glacial acetic acid–methanol (2:100, v/v) and was stored at 4°C. Prior to use, a series of standard solutions over a concentration range of 0.25 to 200 μg/ml was prepared using this stock solution and the same solvent mixture.

2.3.2. Preparation of internal standard solution of II

A stock solution containing 0.4 mg/ml of II was prepared in glacial acetic acid–methanol (2:100, v/v) and stored at 4°C. Prior to use, it was diluted to 20 μg/ml with the same solvent mixture as used for the internal standard solution.

2.3.3. Preparation of calibration standards of I

Calibration standards of I, in the range 5–4000 ng/ml, were prepared as follows. A 20-μl volume of each of the standard solutions of I and 15 μl of the internal standard solution were added into 1.0 ml of blank human plasma in 10-ml glass culture tubes. After adding 1.0 ml of 0.2 *M* sodium hydrophosphate–0.1 *M* citric acid (7.71:12.29, v/v) buffer solution [3] to adjust the pH to 4.0, the spiked plasma was extracted twice using 2 ml of glacial acetic acid–ethyl acetate (1:100, v/v), on a vortex. It is worth noting that, to avoid emulsification and obtain optimum recovery, the plasma should be shaken for 10 min at a frequency of 350 times per min for each extraction. The plasma was then centrifuged at 300 *g* for 10 min. The organic layer was transferred into a tapered glass tube and evaporated to dryness, using nitrogen gas at ambient temperature. The residue was re-dissolved in 200 μl of the mobile phase before chromatographic analysis. The injection volume was 100 μl. The calibration curve was constructed by plotting the concentrations of I versus the peak-area ratio of I to II.

2.3.4. Preparation of human plasma samples

A single dose of I (500 mg) was administered orally to a healthy adult male volunteer who had been on an overnight fast. Blood samples of about 2 ml were drawn using sterile syringes before the

administration of I and at time points from 30 min to 14 days after the administration. The blood samples were immediately transferred to heparinized tubes. After mixing thoroughly with anticoagulant, the samples were centrifuged at 1000 *g* for 15 min, and 1.0 ml of each plasma sample was transferred into plastic cryotubes and frozen at −75°C. After addition of 15 μl of the internal standard solution, II, the plasma samples were treated according to the procedure described in Section 2.3.3 and were then chromatographed. Calibration standards of 20, 200 and 4000 ng/ml were analysed along with each batch of human plasma samples and were plotted over time on a quality control chart. The plasma concentrations obtained were profiled versus time of blood sampling.

2.3.5. Chromatographic conditions

Chromatographic conditions for the determination of I in human plasma were as follows: mobile phase, methanol–water–glacial acetic acid–diethylamine (93:6:1:0.03, v/v); flow-rate, 1 ml/min; wavelength, 335 nm; detector sensitivity (AUFS), 0.02. Although identification and purity examination of the I peak were conducted on a different chromatographic system, all chromatographic conditions were the same.

2.3.6. Selection of a solvent system for extraction

A series of buffer solutions were prepared by mixing a stock solution containing 0.2 *M* boric acid and 0.05 *M* citric acid as "A" and a stock solution of 0.1 *M* sodium phosphate ($Na_3PO_4 \cdot 12H_2O$) as "B" at an A:B ratio of 19.5:0.5 (v/v) for pH 2.0; 15.5:4.5 (v/v) for pH 4.0; 11.8:8.2 (v/v) for pH 6.0; 8.5:11.5 (v/v) for pH 8.0; 5.4:14.6 (v/v) for pH 10.0 and 1.7:18.3 (v/v) for pH 12.0 [4].

A 1-ml volume of the buffer solution, at a pH ranging from pH 2.0 to 12.0, was added into 1 ml of an aqueous solution of I or to 1 ml of I-spiked plasma at a concentration of 4 μg/ml. The mixtures were then extracted twice using 2 ml of glacial acetic acid–ethyl acetate (1:100, v/v) or 6 ml of hexane–dichloromethane (5:4, v/v), followed by the procedure described in Section 2.3.3. The injection volume was 50 μl.

2.3.7. Absolute recovery of I

The absolute recovery of I was obtained by comparing the mean value of the slopes ($n = 6$) of the calibration curves obtained using the calibration standards to the mean value of slopes ($n = 6$) of the calibration curves obtained using the standard solutions [2].

2.3.8. Recovery of internal standard, II

The recovery of II was measured by the ratio of its peak area in the calibration standards prepared (Section 2.3.3.) to that in the standard solution (Section 2.3.2.) [2].

2.3.9. Examination of peak purity

The putative I peak was identified by retention time. The purity of the I peak identified in the chromatograms of spiked plasma and plasma samples drawn after oral administration was checked using on-line UV spectrum.

3. Results and discussion

3.1. Dissolution characteristics of I

Compound I is slightly soluble in water and most of the organic solvents commonly used. It is quite obvious that converting the anion group of I into an ammonium form will help increase its solubility in water. It was found that the dissolution of I in organic solvents, such as methanol and ethyl acetate, could be significantly enhanced by adding 1–2% of glacial acetic acid. This finding was applied to both the optimization of the mobile phase and the selection of a solvent system for the extraction of I from plasma. A reasonable explanation for the large enhancement in dissolution of I is that acetic acid forms an ion-pair with I. This will be further discussed in later sections.

3.2. Optimization of the mobile phase

The chromatographic analysis was carried out on a C_{18} reversed-phase column. The mobile phase used was chosen based on the dissolution properties of I in methanol and its retention behavior on the C_{18} column. The capacity factors (k') of I in a series of

mobile phases is shown in Fig. 2. Pure methanol alone could not elute I from the column. Addition of 1% of glacial acetic acid into methanol as the mobile phase drastically reduced the k' value of I, from infinity to 24.92. With the concentration of glacial acetic acid remaining at 1%, addition of 5% of water to the mobile phase further reduced the k' value to 9.60. However, water could not change the elution strength of methanol for I without the presence of acetic acid. Obviously, the function of water in the mobile phase is to change the polarity and dielectric constant of the latter. Acetic acid forms an ion-pair with I, increasing the solubility of I in the mobile phase. With the addition of water, both the polarity and dielectric constant of the mobile phase increase. As a consequence, the retention of I in the C_{18} stationary phase decreases.

Although acetic acid and water showed their fascinating coordination in the adjustiment of the k' value of I, the eluted peak of I was poorly shaped and broad. It was observed that the addition of a very small amount of diethyl amine remarkably improved the peak shape. As shown in Fig. 2, diethyl amine also enhanced the elution strength of the mobile phase. This enhancement could be contributed to its competition for active electrostatic retention sites on the column, because it can also form an ion-pair with acetic acid. This can also explain the improvement in peak shape gained from its presence in the mobile phase. The optimum composition of the mobile phase, to achieve an adequate separation between I and II as well as to exclude the interference of endogenous substances in human plasma, was found to be 93 parts of methanol, 6 parts of water, 1 part of glacial acetic acid and 0.03 parts of diethyl amine. Chromatograms of plasma samples before and after administration of I with addition of the internal standard are shown in Fig. 3.

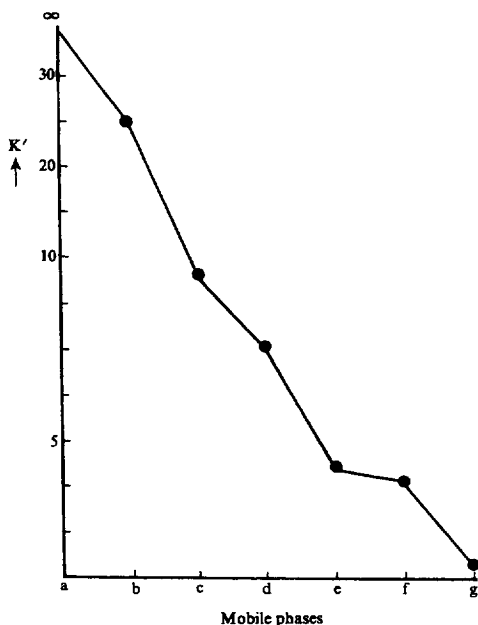

Fig. 2. Capacity factor (k') of benflumetol in different mobile phases. a=Methanol; b=Methanol–glacial acetic acid (99:1, v/v); c=Methanol–water–glacial acetic acid (94:5:1, v/v); d=Methanol–water–glacial acetic acid–diethyl amine (89:10:1:0.01, v/v); e=Methanol–water–glacial acetic acid–diethyl amine (93:6:1:0.01, v/v); f=Methanol–water–glacial acetic acid–diethyl amine (94:5:1:0.01, v/v); g=Methanol–water–glacial acetic acid–diethyl amine (94:5:1:0.02, v/v).

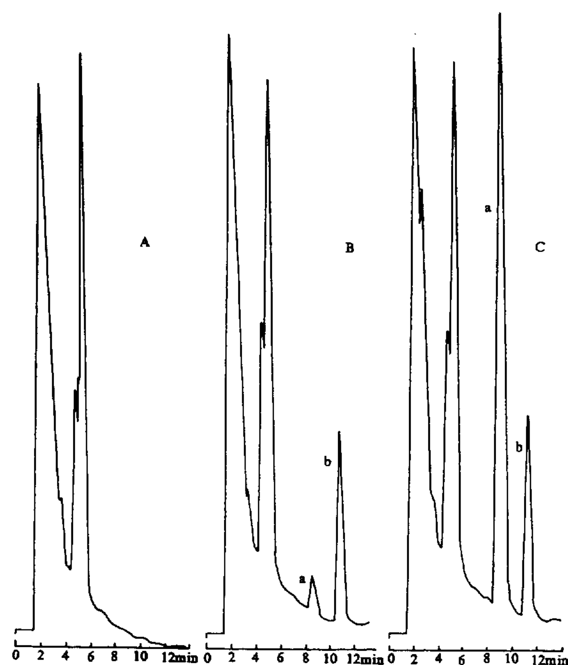

Fig. 3. Chromatogram of human plasma (BIII-11) before and after administration of benflumetol (a) with addition of "8212" (b). A = before administration, B = 2 h after administration, C = 3 h after administration.

3.3. Selection of internal standard

Five homologues of I were examined and II was found to be the best as an internal standard for I, because of its chromatographic properties and spectral characteristics. The high similarity of the homologues to I provided high reliability in the quantitation of the latter but resulted in difficulties in the separation. Under the optimum chromatographic conditions, II can be easily separated from I and no overlapping was observed over the whole concentration range from 5 to 4000 ng/ml. The response factor ratio of II to I was 1:1.22.

3.4. Extraction behavior of I

Since I is an organic base, acidic and neutral solvent systems were chosen for its extraction from water or human plasma at different pHs. Glacial acetic acid–ethyl acetate (1:100, v/v) was chosen as an acidic solvent system. The extraction of I from a spiked plasma into glacial acetic acid–ethyl acetate (1:100, v/v) was quite efficient over a wide pH range, especially in the range from pH 4.0 to 8.0 (see Fig. 4). The extraction behavior of I in spiked plasma and in water was almost the same.

The presence of acetic acid in the solvent system is the key for high extraction efficiency. When compared with glacial acetic acid–ethyl acetate (1:100, v/v), the extraction efficiency of ethyl acetate alone was rather poor, the extraction recovery of I from spiked plasma being only 26%, even at pH 4.0. The results indicated that the enhancement of dissolution of I in ethyl acetate was mainly caused by formation of an ion-pair between acetic acid and I, rather than simply by changes in pH.

A neutral solvent system investigated was hexane–dichloromethane (5:4, v/v). Unlike the glacial acetic acid–ethyl acetate system, the extraction behavior of I from spiked plasma into hexane–dichloromethane (5:4, v/v) was strongly dependent on pH (see Fig. 4). Recovery dropped from about 50% to almost 0% when the pH was increased from 2.0 to 6.0. Furthermore, the extraction behavior of I in water was quite different from that in plasma. While its extraction recovery from plasma was very poor, being only 53% at pH 2.0, I in water showed much higher recoveries (around 70%) over the pH range from 6.0 to 12.0. Since it is known that the protein-binding rate of I in plasma is as high as 95% at pH 7.4, the poor extraction recovery of I in plasma under alkaline condition probably is related to this protein-binding property.

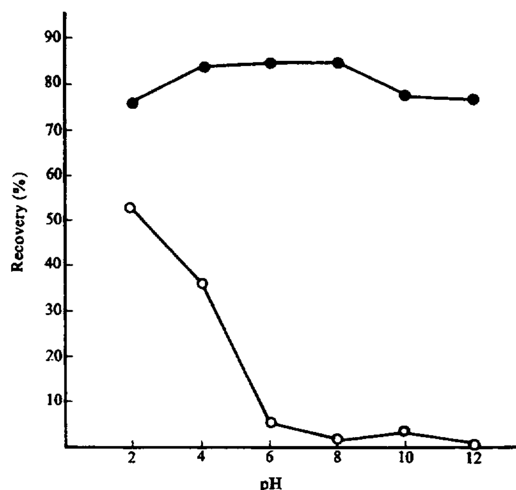

Fig. 4. Influence of pH on the extraction recovery of benflumetol from spiked plasma extracted by different solvent systems. (○) Glacial acetic acid–ethyl acetate (1:100, v/v); (●) hexane–dichloromethane (5:4, v/v).

3.5. Recoveries of I and the internal standard, II

The absolute recovery of I was 92.91% in the concentration range of 5–4000 ng/ml in spiked plasma.

The recovery of internal standard II was 84.85% (n = 24, C.V. = 4.08%) at a concentration of 300 ng/ml in spiked plasma.

3.6. Linearity of calibration curve and detection limit

Due to the wide range of plasma concentrations, it was found to be better to calculate the concentration of I in plasma by two separate calibration curves, one for the lower and the other for the higher concentration range. The best concentration point for division is at about 200 ng/ml. The calibration curve for lower concentrations was linear in the range of 5–200 ng/ml ($y = 0.0044x$, $r = 0.9962$), while that

Table 1
Precision and accuracy of HPLC analysis of benflumetol in plasma

True concentration (ng/ml)	Between-day (n = 6)			Within-day (n = 7)		
	Concentration found (x ± S.D.) (ng/ml)	C.V. (%)	Bias (%)	Concentration found (x ± S.D.) (ng/ml)	C.V. (%)	Bias (%)
5	6.06±1.08	17.85	21.20			
10	11.56±0.95	8.21	15.60	10.75±0.88	8.19	7.50
20	20.01±0.49	2.46	0.05			
50	49.42±1.39	2.82	1.16	50.98±1.91	3.75	1.96
100	99.36±6.14	6.18	0.64			
200	199.50±12.66	6.34	0.25			
500	488.76±23.14	4.73	2.25	502.81±5.86	1.17	0.56
1000	991.86±43.00	4.34	0.81			
4000	4004.86±192.07	4.80	0.12	3904.40±311.87	7.99	2.40

for higher concentrations was linear in the range of 200–4000 ng/ml ($y = 0.0042x - 0.0358$, r = 0.9982).

The detection limit was 11.8 ng/ml. This detection limit was defined by a response signal equal to the mean value of the y-intercepts of calibration curves (n = 6) over the concentration range 5–200 ng/ml as blank signal plus three times the deviation of the blank signal [2].

3.7. Precision and accuracy

The precision (C.V.) and accuracy (bias) of the method were measured by analysis of replicate calibration standards between-day (5–4000 ng/ml) and within-day (10, 50, 500 and 4000 ng/ml). The results are shown in Table 1. The lower concentrations (5 and 10 ng/ml) showed higher coefficients of variation and bias. However, coefficients of variation and bias were good for the concentrations from 20 to 4000 ng/ml. These results are consistent with the detection limit. In fact, the detection limit, measurement precision and accuracy are all dependent on the selection of the detection wavelength. If 234 nm was chosen as the detection wavelength, the detection limit could be lower, and the precision and accuracy of the lower concentrations could be improved. As a matter of fact, the chromatogram of human plasma at 234 nm showed more interfering peaks, therefore the detection wavelength selected was 335 nm, instead of 234 nm.

3.8. Identity and purity of the putative I peak

The retention time and UV spectrum of the putative I peak from either the spiked plasma or plasma samples from the adult volunteer, after administration of I, were identical to those of the reference standard of I, indicating the purity of the putative I peaks. The identification and purity data are shown in Table 2.

3.9. Application of the method

The method was successfully applied to the measurement of I in human plasma samples from ten healthy adult volunteers in a pharmacokinetic study. The mean value of T_{max} was 6.18 h; C_{max} was

Table 2
Retention time and peak purity of benflumetol in different samples

Sample	Retention time (min)	Peak purity
Benflumetol (reference standard)	4.83	1.1
Spiked plasma (4 μg/ml)	4.86	1.1
Plasma after administration of benflumetol	4.86	1.1

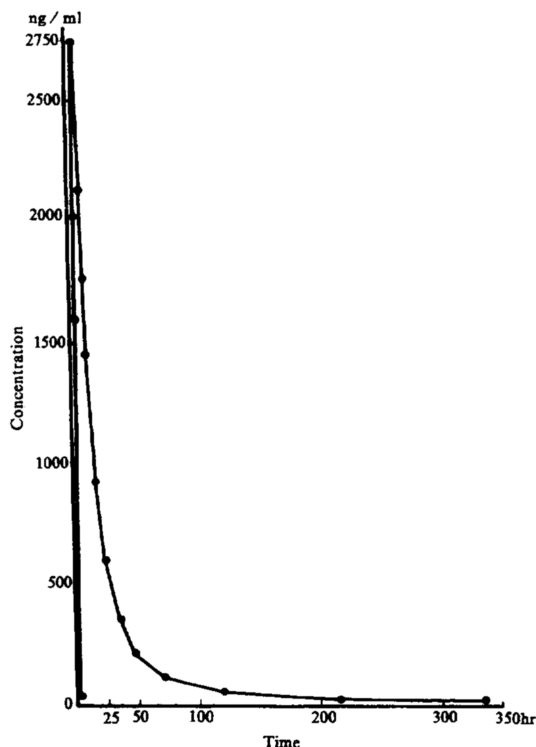

Fig. 5. Plasma concentration–time plots of benflumetol in a healthy volunteer (BIII-11) after oral administration of a single dose of benflumetol (500 mg).

2.0342 μg/ml; and $t_{1/2}\beta$ was 69.5799 h. The plasma concentration–time profile for one of the healthy adult volunteers is shown in Fig. 5. The quality control accompanying each assay batch is given in Table 3, and the control chart is depicted in

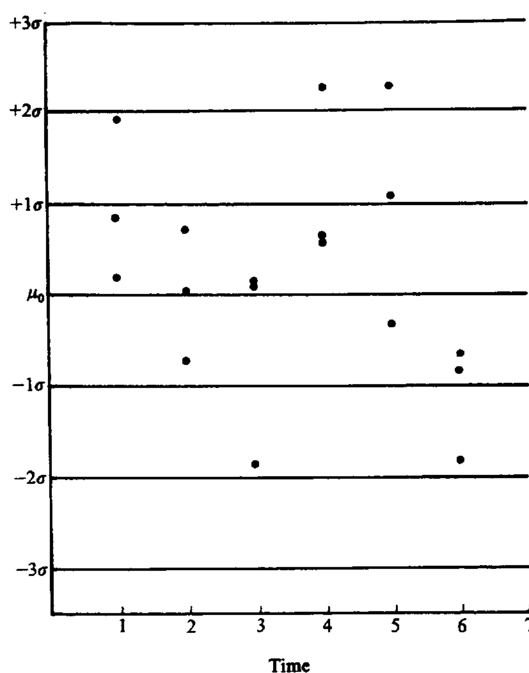

Fig. 6. Quality control chart of calibration standards (20, 200 and 4000 ng/ml).

Fig. 6. All the results verify that the assay procedures were under control.

4. Conclusion

Attributions of the present method are highlighted by: (1) application of the dissolution characteristics

Table 3
Quality control for the analysis of benflumetol in plasma

True concentration (ng/ml)	Warning limit $\mu_o \pm 2\sigma$ (ng/ml)	Action limit $\mu_o \pm 2\sigma$ (ng/ml)	Concentration found in each assay batch (ng/ml)					
			1	2	3	4	5	6
20	20.07 ± 8.78 (11.29 – 28.85)	20.07 ± 13.17 (6.90 – 33.24)	23.77	16.95	11.80	22.53	18.61	17.32
200	199.50 ± 25.32 (174.18 – 224.82)	199.50 ± 37.98 (161.52 – 237.48)	201.57	199.68	201.18	207.32	212.98	176.45
4000	4004.86 ± 384.16 (3620.70 – 4389.02)	4004.86 ± 576.24 (3428.62 – 4581.10)	4369.93	4134.43	4023.79	4440.60	4444.55	3849.12

of I to the extraction of I from human plasma, by the addition of acetic acid to the organic solvent system; (2) coordination of the functions of acetic acid, water and diethyl amine in the mobile phase to separate I and internal standard II as well as to exclude the endogenous substances in human plasma on a C_{18} column; (3) satisfactory recoveries of I and II and (4) controllable procedure process in the assay.

In conclusion, the present method is suitable for pharmacokinetic studies in clinical trials.

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