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Sensitive and rapid LC–ESI-MS method for the determination of trimetazidine in human plasma

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Abstract

A sensitive method, based on liquid chromatography–electrospray ionisation–mass spectrometry (LC–ESI-MS), was developed for the determination of trimetazidine in human plasma. Buflomedil was used as the internal standard (IS). Plasma samples were extracted with a mixture of cyclohexane–diethyl ether (1:1, v/v) and the analytes were chromatographically separated on a phenomenex Luna 5μ C_{18} (2) 100A HPLC column with a mobile phase of 10 mM ammonium acetate buffer solution containing 0.1% acetic acid–methanol (45:55, v/v). The electrospray ionization was employed in a single quadrupole mass spectrometer for the analytical determination. The lower limit of quantification (LLOQ) was 0.5 ng/ml for trimetazidine and the measuring ranges were from 0.5 to 200 ng/ml. The intra- and inter-run standard deviation was less than 4.1% and 7.8%, respectively. The method was successfully applied to study the pharmacokinetics of trimetazidine in healthy Chinese volunteers.

Keywords: Trimetazidine; LC–ESI-MS; Pharmacokinetics

1. Introduction

Trimetazidine dihydrochloride, 1-[(2,3,4-trimethoxyphenyl) methyl]-piperazine (Fig. 1) dihydrochloride, is used as an antianginal drug. In contrast with the classical antianginal drugs, trimetazidine appears to be a very specific treatment, especially to the ischaemic cell. It is only efficacious against the ischaemia-induced loss of membrane functions and its consequences [1]. Several HPLC [2,3], GC–MS [4] and LC–APCI–MS/MS [5] methods have been reported for the determination of trimetazidine in plasma, in which the most sensitive assay is the LC–APCI–MS/MS with an LLOQ of 1.5 ng/ml. The pilot pharmacokinetic study results in our laboratory showed that some of the human plasma levels of trimetazidine on the terminal elimination phase were below 1.5 ng/ml. So, to evaluate the pharmacokinetics of trimetazidine in humans, to develop a more sensitive method for the determination of trimetazidine in human plasma is required. This paper describes the development and validation of a sensitive LC–ESI-MS method with an LLOQ as low as 0.5 ng/ml for the quantification of trimetazidine in human plasma. The method was successfully applied to study the pharmacokinetics of trimetazidine in healthy Chinese volunteers.

2. Experimental

2.1. Materials and reagents

Trimetazidine dihydrochloride (99.2% purity) was supplied by Guangdong Xianqiang Pharmaceutical Co., Ltd. (Guangdong China). Buflomedil Hydrochloride (99.5% purity) was obtained from Jiangsu Institute of Drug Control (Nanjing, China). The test drug formulation was trimetazidine dihydrochloride tablets containing 20 mg of trimetazidine per tablet, which was provided by Guangdong Xianqiang Pharmaceutical Co., Ltd. (Guangdong, China). Methanol of HPLC grade was purchased from Merck KGaA (Darmstadt, Germany). Acetic acid, ammonium acetate, cyclohexane and diethyl ether were analytical grade purity and purchased from Nanjing Chemical Reagent Co., Ltd. (Nanjing, China). Distilled water, prepared from demineralized water, was used throughout the study.

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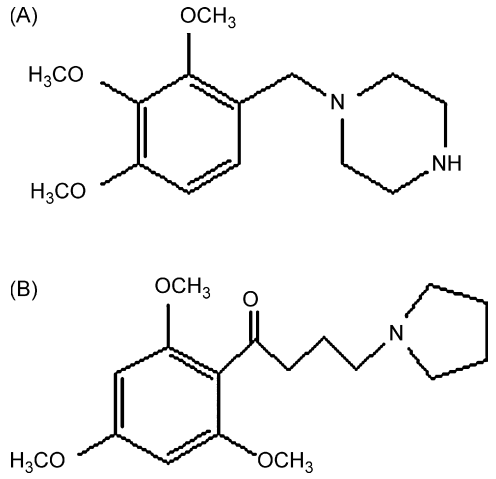

Fig. 1. Chemical structures of trimetazidine (A) and buflomedil (B).

2.2. Instrument and conditions

LC–ESI-MS analyses were performed using an Agilent Technologies Series 1100 LC/MSD VL system (Agilent Technologies, Palo Alto, CA) with a phenomenex Luna 5μ C_{18} (2) 100A HPLC column (250 mm × 4.60 mm, 5 μm, Torrance, CA, USA). The mobile phase was 10 mM ammonium acetate buffer solution containing 0.1% acetic acid–methanol (45:55, v/v) at a flow rate of 1 ml/min. The column temperature was maintained at 25 °C. The electrospray interface was used with the instrument operating in the positive ion mode. The following conditions were used: drying gas (N_2) flow of 10 l/min, drying gas temperature of 300 °C, nebulizer pressure of 40 psi, capillary voltage of 4 kV. The fragmentor voltage was 65 V. LC–ESI-MS was performed in selected-ion monitoring mode selecting the protonated molecule ions $[M+H]^+$ *m/z* 267.3 as the target ion for trimetazidine and $[M+H]^+$ *m/z* 308.4 as the target ion for the IS, respectively.

2.3. Sample preparation

A 1-ml aliquot plasma sample was transferred to a 10 ml glass tube together with 50 μl IS solution (0.4 μg/ml) and 100 μl 1 M sodium hydroxide solutions. After vortex mixing for 10 s, 5 ml of the mixture of cyclohexane–diethyl ether (1:1, v/v) was added, then, the mixture was vortexed for 3 min. Following centrifugation and separation, the organic phase was evaporated to dryness under a nitrogen stream in a water bath of 25 °C. The residue was reconstituted with 120 μl mobile phase and vortexed for 1 min. A 20 μl aliquot was injected into the LC–ESI-MS for analysis.

2.4. Preparation of stock and working solutions

The stock solutions of trimetazidine and IS were prepared at the concentration of 1 mg/ml in methanol. The stock solution of trimetazidine was diluted with methanol to prepare working solutions at the concentrations of 10 μg/ml, 1 μg/ml, 100 ng/ml, and 10 ng/ml. A solution containing 0.4 μg/ml IS was also prepared by further diluting the stock solution of the IS with methanol. All of the solutions were stored at −20 °C.

2.5. Preparation of calibration curves and quality control samples

The calibration samples at concentrations of 0.5, 1, 3, 10, 30, 100, and 200 ng/ml for trimetazidine were prepared daily by spiking different samples of 1 ml blank plasma each with one of the above-mentioned working solutions. The calibration curves were prepared and assayed along with quality control (QC) samples and each batch of clinical plasma samples. The QC samples were prepared at three different concentration levels of 0.8, 18, and 180 ng/ml for trimetazidine in blank plasma.

2.6. Clinical study design and pharmacokinetic analysis

The method was used to determine trimetazidine in plasma samples after administration of a dose of 20 mg trimetazidine to 18 healthy Chinese volunteers in a clinical trial. The clinical study protocol was approved by the Ethics Committee of Tongji Hospital Affiliated to Tongji Medical College of Huazhong University of Science and Technology. All volunteers were given written informed consent to participate in the study according to the principles of the Declaration of Helsinki. Following an overnight fast, each volunteer received one tablet containing 20 mg trimetazidine. The standard meals were provided after 4 h post-dose. The blood samples were collected pre-dose and at 0.5, 1, 1.5, 2, 2.5, 3, 4, 6, 9, 12, 15 and 24 h post-dose. The trimetazidine plasma concentrations of these samples were determined, and the pharmacokinetics of the drug in healthy young Chinese volunteers was evaluated. Model-independent pharmacokinetic parameters were calculated for trimetazidine. The maximum plasma concentration (C_{max}) and the time to it (t_{max}) were noted directly. The elimination rate constant (k_{el}) was calculated by linear regression of the terminal points of the semi-log plot of plasma concentration against time. 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_{0-24} to the last measurable plasma concentration was calculated by the linear trapezoidal rule.

3. Results and discussion

3.1. Mass spectrometry

Because trimetazidine is a weak base, a positive ion-monitoring mode was adopted in the LC–MS assay. Although MS/MS is not available on the instrument used in this experiment, there are still fragment ions [6], deprotonated molecule ions [7,8] and protonated molecule ions [9,10] that can be selected as the target ions of the analytes in the SIM. By adjusting the fragmentor voltage to different values, the different base peaks were obtained. As the fragmentor voltage was set at a lower value, the base peak obtained in the mass spectrum of

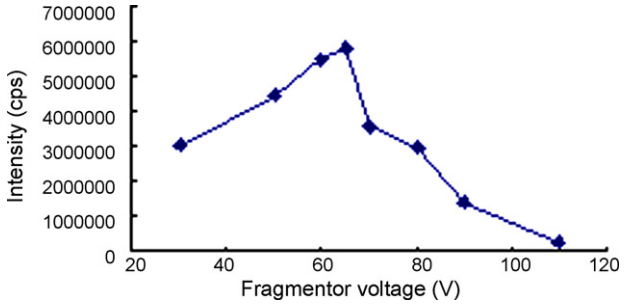

Fig. 2. The intensity of trimetazidine at different fragmentor voltages.

trimetazidine is the protonated molecular ion $[M+H]^+$ at *m/z* 267.3. When the fragmentor voltage exceeded 50 V, the intensity of the fragmentor ion at *m/z* 181.2, which is produced by loss of the group of piperazine ring of trimetazidine, increased obviously, and became the base peak at 80 V. When the fragmentor voltage exceeded 80 V, selecting the fragment ion at *m/z* 181.2 as the target ion can achieve the higher sensitivity. However, this sensitivity was still less than the one achieved by selecting the protonated molecular ion $[M+H]^+$ at *m/z* 267.3 as the target ion with the fragmentor voltage less than 70 V. So the ion at *m/z* 267.3 was finally selected as the target ion for trimetazidine. In order to determine the optimal fragmentor voltage, the intensity of this ion was compared at the fragmentor voltages of 30, 50, 60, 65, 70, 80, 90 and 110 V (see Fig. 2.). The result showed that the highest intensity of the ion at *m/z* 267.3 was obtained by set the fragmentor voltage at 65 V. At this fragmentor voltage, the base peak in the mass spectrum of the IS was the protonated molecular ion $[M+H]^+$ of the IS at *m/z* 308.4. The fragmentations of trimetazidine and the IS are shown in Fig. 3.

3.2. Chromatography

Several experiments show that the employment of an appropriate ratio of ammonium acetate buffer solution in the mobile phase may improve the chromatographic peak shapes [11–13]. So, the different concentrations of ammonium acetate buffer solution at levels of 10 mM, 20 mM and 30 mM were tested in the mobile phase. The results showed that the lowest concentration of 10 mM ammonium acetate buffer tested was able to improve the chromatographic peak shapes of trimetazidine and IS, and resulting in symmetric chromatography peaks. The weak acidic condition of the mobile phase is beneficial to the ionization efficiency of the weak basic compounds in the ESI process of the LC–ESI-MS. Therefore, different concentrations of formic acid at levels of 0.05%, 0.1% and 0.2% and acetic acid at levels of 0.1% and 0.2% were also tested to further optimize mobile phase. Finally, good separation of target compounds was obtained with a mobile phase of 10 mM ammonium acetate buffer solution containing 0.1% acetic acid–methanol (45:55, v/v). Under the present chromatographic conditions, the run time of each sample was 5 min. The retention times were about 3.55 min for trimetazidine and 3.53 min for the IS.

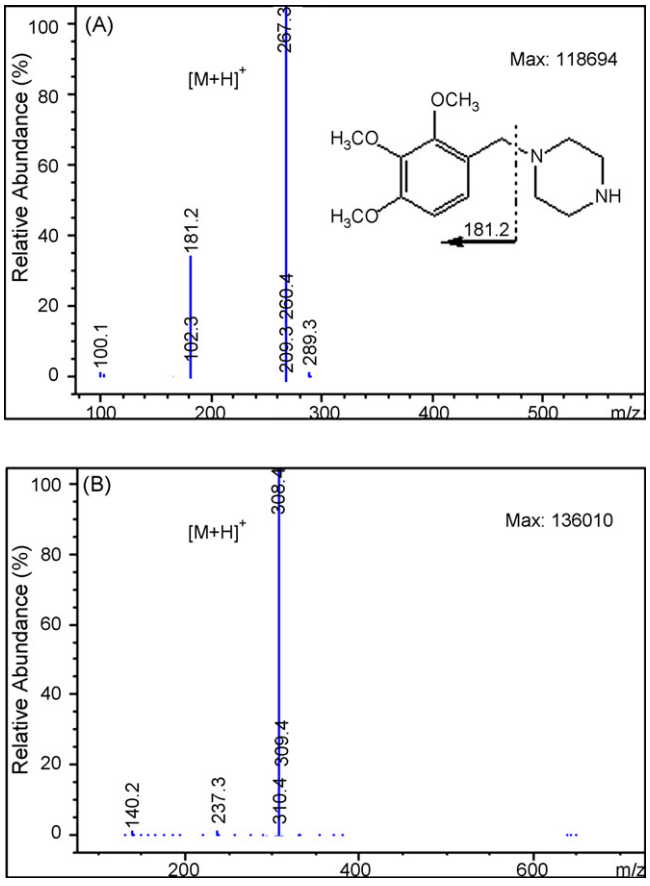

Fig. 3. Mass spectra of the positive ion of trimetazidine (A) and IS (B) at 65 V fragmentor voltage.

3.3. Method validation

3.3.1. Selectivity, calibration curve and lower limits of quantification

The selectivity of the method was evaluated by analyzing six different batches of blank human plasma. All samples were found to have no interferences at the retention times of the analytes. Fig. 4 shows the typical chromatograms of blank plasma; blank plasma spiked with trimetazidine (200 ng/ml) and the IS; LLOQ for trimetazidine in human plasma (0.5 ng/ml) and the IS; a plasma sample from a healthy volunteer 6 h after an oral administration.

The linearity of calibration curve was tested over the concentration range of 0.5–200 ng/ml, using weighted least squares linear regression (weighting factor was $1/C^2$). The calibration curve for trimetazidine had a slope of 0.01679 ± 0.00058, an intercept of -0.0008997 ± 0.0003013 and $r = 0.9991 \pm 0.0005$ (mean ± S.D., $n = 3$).

The lower limit of quantitation (LLOQ) of the assay was defined as the lowest concentration in the standard curve that can be quantitated with accuracy within ±20% and the standard deviation was within 20% [14]. The present LC–ESI-MS method offered an LLOQ of 0.5 ng/ml for trimetazidine, which was established using five samples independent of calibrator standards (see Table 1).

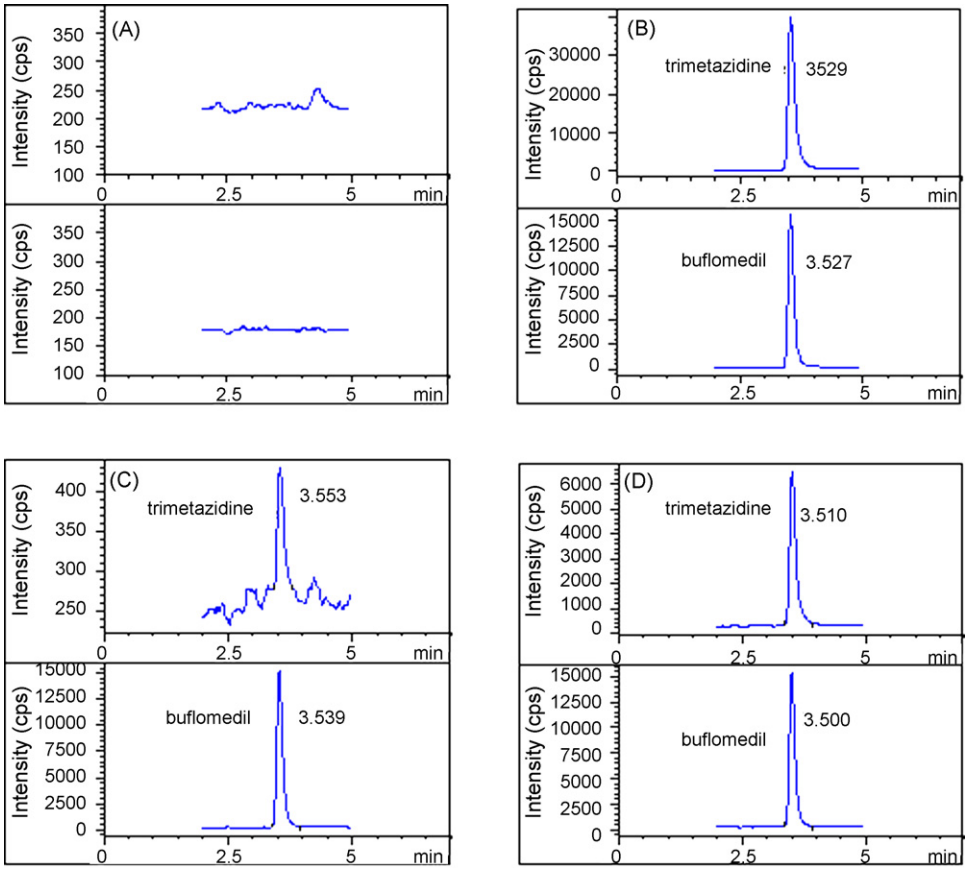

Fig. 4. Typical SIM chromatograms of blank plasma (A), plasma spiked with trimetazidine (200 ng/ml) and IS (B), LLOQ for trimetazidine in plasma (0.5 ng/ml) and IS (C), plasma obtained from a volunteer at 6 h after oral administration of 20 mg trimetazidine, the plasma concentration of trimetazidine was estimated to be 23.32 ng/ml (D).

3.3.2. Precision and accuracy

Validation samples were prepared and analyzed on three consecutive days (one run per day) to evaluate the accuracy and the intra-run and inter-run precision of the analytical method. The accuracy as well as the intra-run and inter-run precision of the method was determined by analyzing five replicates at 0.8, 18, and 180 ng/ml of trimetazidine along with one standard curve on each of 3 days. Assay precision was calculated using the relative standard deviation (R.S.D.%). The accuracy is the degree of closeness of the determined value to the nominal true value under prescribed conditions. Accuracy is defined as the relative deviation in the calculated value (E) of a standard from that of its true value (T) expressed as a percentage (RE%). It was calculated by using the formula: $RE\% = (E-T)/T \times 100$. The results (see Table 2) demonstrated that the method was accurate and precise.

Table 1
Accuracy and precision for the analysis of LLOQ ($n = 5$)

Added C (ng/ml)	Found C (ng/ml)	Mean (ng/ml)	R.S.D. (%)	RE (%)
0.5155	0.4486			−13.0
0.5155	0.4329			−16.0
0.5155	0.5171	0.4666	9.0	0.3
0.5155	0.5064			−1.8
0.5155	0.4282			−16.9

Note: R.S.D., relative standard deviation; RE, relative error; n, number of replicates.

3.3.3. Recovery and stability

The mixture of cyclohexane–diethyl ether (1:1, v/v) that was chosen as the extraction solvent can not only eliminate the interference of endogenous substances, but also meet the requirement of sensitivity for the assay. Because trimetazidine is a weak base, the extraction recovery of trimetazidine can be improved by raising the pH of the plasma samples with sodium hydroxide solutions. Therefore, a 100 μl aliquot of 1 M sodium hydroxide solutions was added to 1 ml plasma sample before extraction. The extraction recovery of the assay, determined at three concentrations of 0.8, 18, and 180 ng/ml were 55.2%, 61.8% and 59.2%, respectively.

Table 2
Precision and accuracy of the assay for the determination of trimetazidine in human plasma ($n = 3$ runs, five replicates per run)

Added to plasma (ng/ml)	Mean measured concentration (ng/ml)	RE (%)	Intra-assay R.S.D.%	Inter-assay R.S.D.%
0.8248	0.7453	−9.6	3.1	7.8
18.56	17.08	−8.0	4.1	7.5
185.6	175.2	−5.6	2.5	3.4

Table 3
Stability data of trimetazidine in human plasma under various storage conditions ($n = 3$)

Storage conditions	Added C (ng/ml)	Found C (ng/ml)	Intra-run R.S.D. (%)	RE (%)
Room temperature for 8 h	0.8248	0.8197	6.1	−0.6
	185.6	167.4	0.5	−9.8
Three freeze–thaw cycles	0.8248	0.7514	7.7	−8.9
	185.6	179.6	2.5	−3.2
4 weeks at −20 °C	0.8248	0.8093	4.5	−1.9
	185.6	183.7	1.4	−1.0

Table 4
Matrix effect evaluation of trimetazidine and IS in human plasma ($n = 5$)

Samples	Spiked concentration (ng/ml)	A (mean ± S.D.)	B (mean ± S.D.)	Matrix effect (%)
Trimetazidine	0.8	3992 ± 195	3801 ± 562	105
	18	92,833 ± 4288	90,249 ± 12,517	103
	180	773,705 ± 43,468	711,424 ± 10,866	109
IS	400	1,59,248 ± 8799	1,46,066 ± 23042	109

The stability of trimetazidine in plasma was studied under a variety of storage and handling conditions at low (0.8 ng/ml) and high (180 ng/ml) concentration levels. The short-term temperature stability was assessed by analyzing three aliquots of each of the low- and high-concentration samples that were thawed at room temperature and kept at this temperature for 8 h. Freeze–thaw stability (−20 °C in plasma) was checked through three cycles. Three aliquots at each of the low and high concentrations were stored at −20 °C for 24 h and thawed unassisted at room temperature. When completely thawed, the samples were refrozen for 24 h under the same conditions. The freeze–thaw cycles were repeated three times, and then analyzed on the third cycle. The long-term stability was determined by analyzing three aliquots of each of the low and high concentrations stored at −20 °C for 4 weeks. The results in Table 3 showed that no significant degradation of trimetazidine was observed under the tested conditions.

3.4. Matrix effect

The matrix effect (ME) was defined as the direct or indirect alteration or interference in response due to the presence of unintended analytes or other interfering substances in the sample [14]. It was examined by comparing the peak areas of the analytes and IS between two different sets of samples. In set 1, analytes was resolved in the blank plasma sample's reconstituted solution, and the obtained peak areas of analytes were defined as A. In set 2, analytes was resolved in mobile phase, and the obtained peak areas of analytes were defined as B. ME was calculated by using the formula: ME (%) = $A/B \times 100$. The matrix effect of the method was evaluated at three trimetazidine concentration levels of 0.8, 18 and 180 ng/ml and the IS concentration level of 400 ng/ml. Five samples at each level of the analytes were analyzed. The blank plasma samples used in this study were from five different batches of human blank plasma. If the ME values exceed the range of 85–115%, an exogenous matrix effect is implied. As shown in Table 4, the results obtained were well within the acceptable limit, it indicated that there was no matrix effect of the analytes observed in this study.

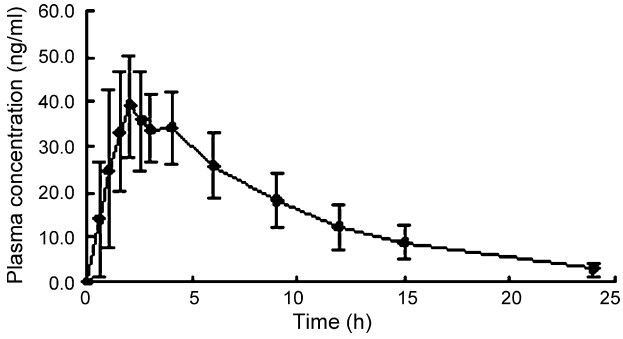

Fig. 5. Mean plasma concentration- time profile of trimetazidine after an oral administration of 20 mg trimetazidine to 18 healthy volunteers.

3.5. Applications

The method described above was successfully applied to determine the plasma concentration of trimetazidine. After an oral administration of 20 mg trimetazidine to 18 volunteers, the main pharmacokinetic parameters of trimetazidine were estimated. The mean plasma concentration–time curve of trimetazidine was shown in Fig. 5. The main pharmacokinetic parameters of trimetazidine in 18 volunteers were calculated

Table 5
Mean pharmacokinetic parameters of trimetazidine for 18 volunteers after oral administration of 20 mg trimetazidine tablet

Parameters	Test tablet
k_{el} (h^{-1})	0.127 ± 0.020
$t_{1/2}$ (h)	5.9 ± 1.4
C_{max} (ng/ml)	44.4 ± 7.3
t_{max} (h)	2.1 ± 0.9
AUC_{0-24} (h ng/ml)	370.8 ± 91.3

and summarized in Table 5. Krishnaiah et al. [3] described the pharmacokinetic profiles of trimetazidine in India healthy volunteers. The pharmacokinetic parameters of trimetazidine were generally similar in India and Chinese volunteers.

4. Conclusions

The results obtained in this study demonstrate that the LC–ESI-MS method is suitable for accurate quantification of trimetazidine in human plasma and has a high sensitivity with an LLOQ of 0.5 ng/ml. No significant interference and matrix effect caused by endogenous compounds were observed. The method is suitable for the pharmacokinetic study and bioavailability evaluation of trimetazidine in human subjects.

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