Department of Biopharmaceutics, School of Pharmacy, Shenyang Pharmaceutical University, Shenyang, China

Quantification of trimetazidine in human plasma by liquid chromatography-electrospray ionization mass spectrometry and its application to a bioequivalence study

Z. B. Wang, J. Sun, R. Rong, J. L. Tang, Z. G. He

Received March 16, 2006, accepted April 20, 2006

Prof. Zhonggui He, Mailbox 59#, Department of Biopharmaceutics, School of Pharmacy, Shenyang Pharmaceutical University, 103 Wenhua Road, Shenyang 110016, China
hezhgui@mail.sy.ln.cn

Pharmazie 62: 27–30 (2007) *doi: 10.1691/ph.2007.1.6051*

A rapid, sensitive and specific liquid chromatography-electrospray ionization mass spectrometric (LC-ESI-MS) method has been developed for the quantification of trimetazidine in human plasma. The analyte and the internal standard (pseudoephedrine) were extracted from plasma samples with *n*-hexane-dichloromethane (1 : 1, v/v) and analyzed on a C_{18} column. The chromatographic separation was achieved within 3.5 min using the mobile phase consisting of methanol/0.05% formic acid (80 : 20, v/v) and the flow rate was 1.0 ml/min. Ion signals m/z 181.0 and 148.0 were measured in the positive mode for trimetazidine and pseudoephedrine, respectively. The calibration curves were linear within the range of 0.4∼120 ng/ml. The lower limit of quantification (LLOQ) was 0.4 ng/ml with 0.5 ml plasma sample. The intra- and inter-day precisions were lower than 12% in terms of relative standard deviation (RSD). The inter-day relative error (RE) as determined from quality control samples (QCs), ranged from −1.4% to 3.3%. This validated method was successfully applied to the bioequivalent evaluation of two brands of trimetazidine tablets in 20 healthy volunteers.

1. Introduction

Trimetazidine, 1-(2,3,4-trimethoxybenzyl) piperazine dihydrochloride, is an effective and well-tolerated antianginal agent that has a cytoprotective action during ischemia and provides symptom relief and functional improvement in patients with angina pectoris (Karen and Greg 1999). A recent survey revealed that several methods were available for the determination of trimetazidine in biological fluids. HPLC-UV was used for its quantitation in blood and urine (Gaillard et al. 1997; Krishnaiah et al. 2002). HPLC with fluorescence detection was proposed for the assay of trimetazidine which was detected as a fluorescent dansyl derivative (Courte and Bromet 1981). HPLC coupled to electrochemical detection was reported as another alternative for determination of trimetazidine in plasma samples (Brai et al. 1999). The drug was also determined by gas chromatography (Fay et al. 1989). LC-MS was developed as a quantitative method for assaying of trimetazidine from plasma samples (de Jager et al. 2001). The sample preparation procedure was relatively complicated, and it is recommended to avoid the use of internal standards for reproducibility concerns. LC-MS-MS was used for the analysis of trimetazidine in plasma samples (Andrei et al. 2004). Trimetazidine and internal standard were isolated from plasma by protein precipitation with trifluoroacetic acid. The method was validated with a quantitation limit for trimetazidine of 1.5 ng/ml, but the adopted internal standard was not easily available.

In this paper, we report a rapid and more sensitive liquid chromatography-electrospray ionization mass spectrometric (LC-ESI-MS) method for reliable and sensitive quantification of trimetazidine in human plasma.

2. Investigations, results and discussion

2.1. *Method validation*

Trimetazidine and pseudoephedrine (internal standard, I.S.) were well separated under the experimental conditions, with retention times being 2.32 and 2.81 min, respectively (Fig. 1B). No endogenous interferences were found at the corresponding retention times of drug and I.S. (Fig. 1A), and both compounds eluted as completely resolved peaks in the human plasma sample at a single 20 mg oral dose of trimetazidine (Fig. 1C).

Calibration curves for the plasma assay developed with peak-area ratio (Y) of trimetazidine to internal standard versus drug concentration (X) were found to be linear over the concentration range of 0.4∼120.0 μg/L. The linear regression equation of the calibration curve was $Y = 5.430 \times 10^{-3} + 2.953 \times 10^{-2}$ X, with $r = 0.999$. The LLOQ at which both precision and accuracy were less than 12%, was 0.4 ng/ml when using 0.5 ml plasma sample.

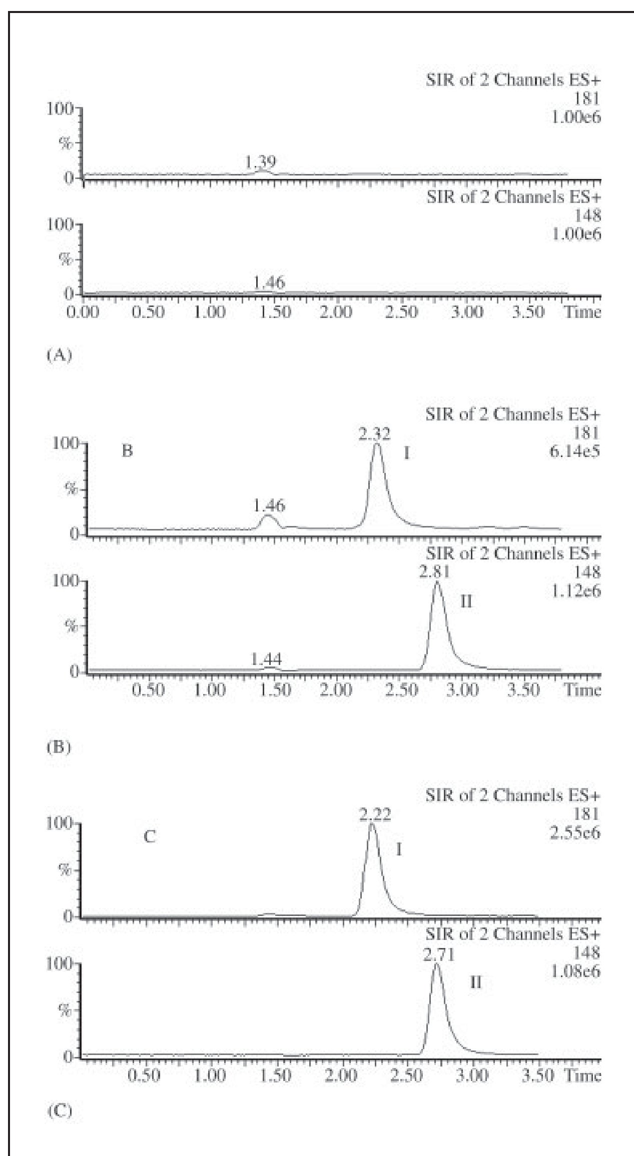

Fig. 1: Representative SIR chromatograms of trimetazidine (I) and pseudoephedrine (II) in human plasma determined by LC-ESI-MS method. (A) A blank plasma sample; (B) a blank plasma sample spiked with trimetazidine at the LLOQ of 20 ng/ml and pseudoephedrine (20 ng/ml); (C) a plasma sample collected at 2 h after oral administration of 20 mg trimetazidine to a healthy volunteer

Accuracy and precision of the method were evaluated by a one-way analysis of variance (ANOVA) based on the data from quality control samples (QCs) in three validation runs. The accuracy was determined by calculating the percentage deviation observed in the analysis of QCs and expressed in the relative error. The intra- and inter-run precision was expressed as the relative standard deviation. The intra- and inter-run precision was less than 16.0% and 7.0%, respectively, and the accuracy was within ±3.33% (Table 1), indicating the acceptable accuracy and precision of the method developed.
Extraction recoveries of trimetazidine at concentrations of 1.0, 20 and 80 ng/ml (n = 6) were determined to be 65.9%, 70.0%, and 75.8%, respectively. The extraction recovery of pseudoephedrine was measured as 71.5% at the concentration of 20.0 ng/ml. Extraction recoveries for trimetazidine and pseudoephedrine were consistent, precise and reproducible throughout the validation experiments and were within the acceptance criteria.

Table 1: Precision and accuracy for the analysis of trimetazidine in human plasma

Added conc. (ng/ml)	Found conc. (ng/ml)	Intra-run RSD (%)	Inter-run RSD (%)	Relative error (%)
1.0	1.01	11.12	6.04	0.50
20.0	20.67	4.81	3.82	3.33
80.0	78.86	2.51	3.93	−1.43

(in = 3 days, six replicates per day)

Table 2: Stability data of trimetazidine in human plasma under various storage conditions

Storage conditions	Added conc. (ng/ml)	Found conc. (ng/ml)	Inter-run RSD (%)	Relative error (%)
Three freeze-thaw cycles	1.0	0.96	8.5	−4.0
	20.0	19.93	5.1	−0.3
	80.0	76.23	2.2	−4.7
24 h at ambient temperature	1.0	0.96	9.4	−4.3
	20.0	19.67	6.4	−1.7
	80.0	76.93	2.3	−3.8

(n = 3)

Trimetazidine is an alkaline compound, so plasma samples were basified in order to improve the extraction recovery of trimetazidine. Several alkalization reagents were investigated, including sodium hydroxide, sodium carbonate and sodium bicarbonate with different concentrations. Finally, sodium hydroxide (2 mol/l) was chosen.
Stability experiments showed that no significant degradation occurred at ambient temperature for 24 h and during the three freeze-thaw cycles for trimetazidine plasma samples. Stability data of trimetazidine in human plasma are shown in Table 2. Standard solutions of trimetazidine and pseudoephedrine were shown to remain stable for at least 20 days at 4 °C. The results were obtained by comparing with those of solutions freshly prepared. The concentration deviations were within ±5%.

2.2. Bioequivalence evaluation

The mean blood concentration-time profiles of trimetazidine after a single 20 mg oral dose of trimetazidine test and reference tablets are shown in Fig. 2. The maximum

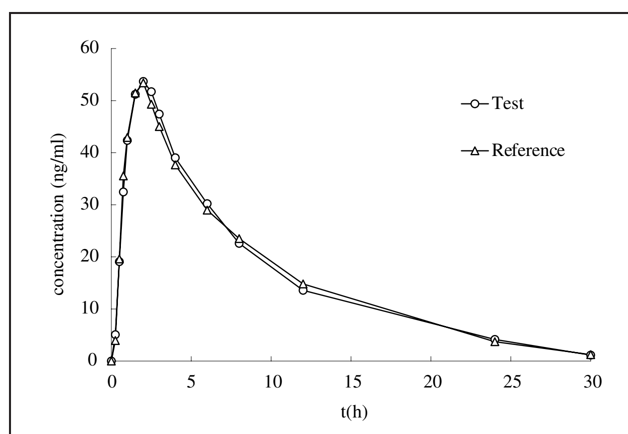

Fig. 2 Mean plasma concentration-time profile of trimetazidine after oral administration of 20 mg trimetazidine tablets to 20 healthy volunteers (each value represents the mean + SD, n = 20)

blood concentrations (C_{max}) were 60.5 ± 13.4 ng/ml and 61.3 ± 14.7 ng/ml for test and reference preparations, respectively. Moreover, the times to reach the peak concentrations (T_{max}) of two preparations were obtained at 1.9 ± 0.5 h and 1.9 ± 0.5 h for test and reference tablets, respectively. The relative bioavailability of test tablets was $100 \pm 10\%$, compared to reference preparation. There is no statistically significant difference in the main pharmacokinetic parameters, and two formulations were considered to be bioequivalent.

3. Experimental

3.1. Chemicals and reagents

Test tablets (trimetazidine dihydrochloride tablets) were supplied by Beijing Wansheng Pharm. Co., Ltd. (each contains 20 mg of the drug). Reference tablets (Vasorel®) were purchased from Les Laboratoires Servier (each contains 20 mg trimetazidine dihydrochloride, Gidy, France). Reference standards of trimetazidine and pseudoephedrine were provided by Beijing Wansheng Pharm. Co., Ltd. HPLC-grade methanol, *n*-hexane and dichloromethane were obtained from Concord Tech. Co. (Tianjin, China). All other reagents were of analytical grade. Blank human plasma was purchased from Shenyang Blood Donor Service (Liaoning, China). All other reagents were of analytical grade. Distilled water, prepared from demineralized water, was used throughout the study.

3.2. LC-MS conditions

The chromatographic system consisted of a Waters 1525 pump, a ZQ2000 micromass spectrometer equipped with an electrospray ionization (ESI) source and Masslynx 4.0 Software (Waters Corporation, USA). The chromatographic separation was achieved on a Diamonsil C_{18} analytical column (200 × 4.6 mm, 5 μm; Dikma, China) with a Security Guard C_{18} guard column (4 × 3.0 mm, Phenomenex, CA, USA). Methanol/0.05% formic acid (20 : 80, v/v) degassed by sonication before use, was employed as the mobile phase and delivered at a flow-rate of 1.0 ml/min with split ratio of 3 : 1.
The mass spectrometer was operated in the positive mode. Quantification was performed using selected reaction monitoring (SRM) of the transitions of m/z 309 → m/z 181 for trimetazidine and m/z 166 → m/z 148 for pseudoephedrine, respectively, with a scan time of 0.2 s per transition. Fig. 3 shows the product ion spectra of $[M + H]^+$ of trimetazidine and pseudoephedrine.
The following parameters were optimized: capillary voltages, 2.8 KV; cone voltages, 32 V; source temperature, 105 °C; desolvation temperature, 400 °C and delivered at a flow rate of 350 l/h.

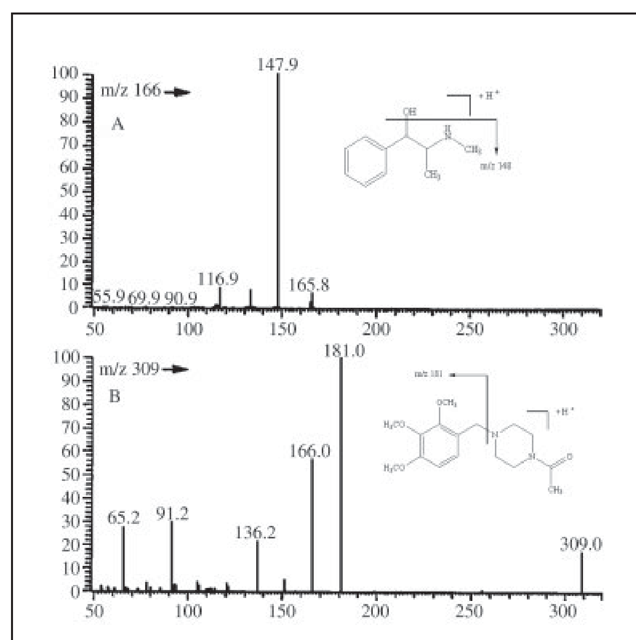

Fig. 3 Full-scan product ion spectra of $[M + H]^+$ of trimetazidine (A) and pseudoephedrine (B)

3.3. Preparation of standards and quality control samples

Stock solutions of trimetazidine were prepared in methanol at the concentration of 100 μg/ml. Stock solutions of pseudoephedrine were prepared in methanol at the concentration of 100 μg/ml and diluted to 100 ng/ml with methanol. Calibration curves were prepared by spiking 100 μl of the appropriate standard solution to 0.5 ml of blank plasma. Final concentrations in plasma samples were 0.4, 1, 2, 10, 20, 40, 80, 120 ng/ml for trimetazidine. The QCs were prepared in the same way at the concentrations of 1, 20, 80 ng/ml, respectively. The spiked plasma samples (standards and quality controls) were treated following extraction on each analytical batch along with the unknown samples.

3.4. Sample preparation

After spiking 100 μl mobile phase, 100 μl of I.S. solution and 200 μl of 2 M Sodium hydroxide, into 0.5 ml of plasma samples, and then the mixed samples were extracted with 3 ml of *n*-hexane-dichloromethane (1 : 1, v/v). The mixture was vortexed for approximate 3 min, then centrifuged for 10 min at 3500 rpm. The upper organic layer was removed and evaporated to dryness at 40 °C under a gentle stream of nitrogen. The residue was reconstituted in 100 μl of mobile phase and vortexed for 1 min. A 20 μl aliquot of the resulting solution was injected onto the LC-MS system for analysis.

3.5. Method validation

Plasma samples were quantified using the ratio of the peak-area of trimetazidine to that of I.S. as the assay parameter. Peak area ratios were plotted against trimetazidine concentrations and standard curves in the form of $y = A + Bx$ were calculated using weighted ($1/x^2$) least squares linear regression.
To evaluate linearity, plasma calibration curves were prepared and assayed in duplicate on three separate days. The accuracy and precision were also assessed by determining QCs at three concentration levels on three different validation days. The accuracy was expressed by relative error (RE) and the precision by relative standard deviation (RSD).
Absolute recoveries of trimetazidine at three QC levels were determined by assaying the samples as described above and comparing the mean peak area of extracted samples with the mean peak area of unextracted samples at the respective concentrations.
Stability of plasma samples subjected to three freeze-thaw cycles and 24 h at ambient temperature was assessed by analyzing replicates ($n = 3$) of QCs. The results were compared with those of QC samples freshly prepared, and the percentage of concentration deviation was calculated.

3.6. Bioequivalence study

The open, two-period, randomized crossover protocol was approved by the Ethics Committee of People's Hospital of Liaoning Province (China). A favorable written informed consent was obtained before each subject's participation in the clinical trial. In addition, each volunteer had been informed of the purpose protocol and risks of the study. The clinical pharmacokinetic study conformed to the principles of the Declaration of Helsinki. The mean age of 20 male healthy volunteers was 22.1 ± 1.3 years, and the mean weight was 60.2 ± 5.9 kg. Serial blood samples (4 ml) from a suitable antecubital vein were collected into sodium heparin-containing tubes before and 0.25, 0.5, 0.75, 1.0, 1.5, 2.0, 2.5, 3.0, 4.0, 6.0, 8.0, 12.0, 24.0, 30.0 h after oral administration of trimetazidine. Plasma was obtained by centrifugation for 10 min at 3500 rpm and stored frozen at −20 °C until analysis.
Pharmacokinetic parameters were determined from the plasma concentration-time data. The maximum blood concentration (C_{max}) and the time to reach maximum concentration (T_{max}) were determined by a visual inspection of the experiment data. The area under the blood concentration-time curve from time zero to the last measurable blood concentration point ($AUC_{0-30\,h}$) was calculated by the linear trapezoidal rule. The relative bioavailability (F%) was calculated by $F\ (\%) = AUC_{Test}/AUC_{Reference}$. An unpaired student's t-test was used to determine any significant difference between the test and the reference formulations. The differences were considered to be significant at $P < 0.05$.

References

Bari VR, Dhorda UJ, Sunderesan M (1999) Trace determination of trimetazidine hydrochloride in human blood plasma by HPLC using electrochemical detection. Indian Drugs 36: 289–292.
Courte S, Bromet N (1981) Trace determination of trimetazidine in plasma by high performance liquid chromatography using fluorescence detection. J Chromatogr B: Biomed Sci and Appl 224: 162–167.
de Jager AD, Sutherland FCW, Badenhorst D, Hundt HKL, Swart KJ, Scanes T, Hundt AF (2001) High throughput assay method for the quantitation of trimetazidine in human plasma by LC/MS with selected reaction monitoring. J liq Chromatogr Rel Technol 24: 2121–2132.

Fay L, Michel G, Groupie P, Harpey C, Prost M (1989) Determination of trimetazidine in biological fluids by gas chromatography mass spectrometry. J Chromagr B: Biomed Sci Appl 82: 198–205.
Gaillard Y, Pepin G (1997) Use of high performance liquid chromatography with photodiode array UV detection for the creation of a 600-compound library. Application to forensic toxicology. J Chromatogr A 763: 149–163.
Krishnaiah YS, Karthikeyan RS, Bhaskar P, Satyanarayana V (2002) Bioavailability studies on guar gum-based three-layer matrix tablets of trimetazidine dihydrochloride in human volunteers. J Control Release 83: 231–239.
McClellan KI, Plosker GL (1999) A review of its use in stable angina pectoris and other coronary conditions. Drugs 58: 143–151.
Medvedovici A, Florin Albu F, Cristina Georgită C, David V (2005) Nonextractive procedure followed by LC/APCI MS/MS analysis of trimetazidine in plasma samples for assessing bioequivalence of immediate/modified release formulations. Biomedi Chromatogr 19: 549–555.