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Development and validation of a sensitive LC–MS/MS assay for simultaneous quantitation of ranolazine and its three metabolites in human plasma

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ABSTRACT

A rapid, sensitive and reliable LC–MS/MS method was developed and validated for the simultaneous determination of ranolazine and its three metabolites, CVT-2514, CVT-2738, and CVT-4786, in human plasma. The plasma samples were prepared by protein precipitation. Chromatographic separation was achieved on a Gemini C_{18} column (50 mm × 2.0 mm, 5 μm) using methanol: 5 mM ammonium acetate as the mobile phase with gradient elution. Mass detection was carried out by electrospray ionization in both positive and negative ion multiple reaction monitoring (MRM) modes. The calibration curves were linear over a concentration range of 4–2000 ng/mL for ranolazine, 4–1000 ng/mL for CVT-2514 and CVT-2738 and 8–1000 ng/mL for CVT-4786. The intra-day and inter-day accuracy and precision were within the acceptable limits of ±15% at all concentrations. The method was successfully applied for the simultaneous estimation of ranolazine and its three metabolites in human plasma from a clinical pharmacokinetics study.

1. Introduction

Chronic angina pectoris remains a widespread health problem despite advances in techniques and therapies [1]. Ranolazine (Fig. 1) is a novel antianginal agent approved for the treatment of chronic stable angina. In contrast to other antianginal drugs, such as beta-blockers and calcium channel blockers, ranolazine exerts its effects through the inhibition of the transcellular late sodium current. Ranolazine has negligible effects on blood pressure and heart rate, which may proved to be the major advantage of the drug [2,3].

Ranolazine is extensively metabolized, predominantly by enzymes of the cytochrome P450 (CYP) 3A family and to a lesser extent by CYP2D6, with 5–10% excreted unchanged by the kidneys. Among its metabolites, CVT-2514, CVT-2738, and CVT-4786 (Fig. 1) occur at concentrations of >10% at steady state compared with the parent compound [4]. Therefore, developing sensitive, accurate, and high-resolution methods for the determination of these substances in the human plasma is necessary.

Several methods, including HPLC and LC–MS/MS, have been applied for the determination of ranolazine in the plasma [5–9]. However, very few analytical methods for simultaneously quantitating ranolazine and its metabolites in human plasma have been published in the literature to date. Penman et al. developed an LC–MS assay to identify the metabolites of ranolazine in human plasma [10], and Herron et al. described another LC/MS method to determine ranolazine and semi-quantitate the observed metabolites [11]. Unfortunately, these two articles did not provide detailed descriptions of the methods used. To the best of our knowledge, no other published reports are available with regard to the quantitation of ranolazine and its metabolites.

The assay described in the present article is a validated, sensitive analytical tool capable of simultaneously measuring ranolazine and its three main metabolites CVT-2514, CVT-2738, and CVT-4786 in the human plasma using LC–MS/MS. This method has been successfully applied to assess the pharmacokinetics of ranolazine in a clinical study.

2. Experimental

2.1. Reagents and materials

Ranolazine (99.2% purity), CVT-2514 (>98% purity), CVT-2738 (>99% purity) and CVT-4786 (>95% purity) were provided by Fujian Fukang Pharmaceutical Co., Ltd. (Fujian, China). Fentanyl (100% purity, IS for ranolazine and CVT-2514) was purchased from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). Glipizide (100% purity, IS for CVT-2738) and lipoic acid (99.6% purity, IS for CVT-4786) were provided by

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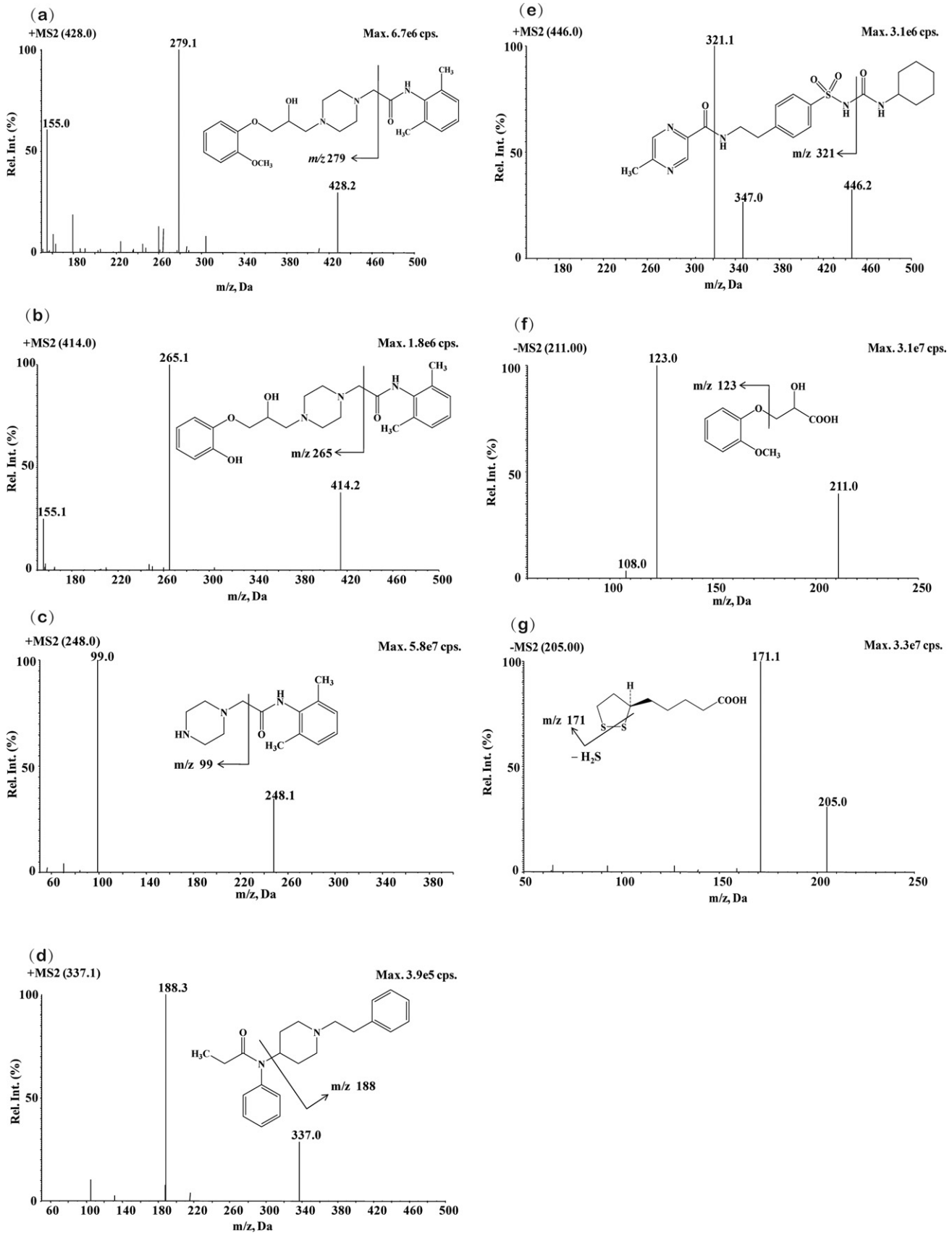

Fig. 1. Product ion mass spectra of $[M+H]^+$ ion of (a) ranolazine, (b) CVT-2514, (c) CVT-2738, (d) fentanyl and (e) glipizide; $[M-H]^-$ ion of (f) CVT-4786 and (g) lipoic acid.

Taizhou Highsun Pharmaceutical Co., Ltd. (Zhejiang, China) and Shandong Qidu Pharmaceutical Co., Ltd. (Shandong, China), respectively. Ammonium acetate of HPLC-grade was purchased from ROE Scientific Inc. (Newmark, DE, USA). HPLC-grade methanol was purchased from Sigma–Aldrich (St. Louis, MO, USA) and HPLC-grade acetonitrile was purchased from Merck (Darmstadt, Germany). The water (18 MΩ) was purified using the Millipore Milli-Q Gradient Water Purification System (Molsheim, France).

2.2. Instrumentation

The liquid chromatography system utilized a Shimadzu (Kyoto, Japan) LC-30AD pump and a Shimadzu SIL-30AC autosampler. Mass spectrometric detection was conducted on an AB SCIEX QTRAP 5500 system (Applied Biosystems/MDS Analytical Technologies, Foster City, CA, USA) equipped with a Turbo Ionspray® ionization interface. The data acquisition software used was Analyst™ version 1.5.

2.3. Chromatographic conditions

Chromatographic separation was performed on a Gemini C_{18} column (50 mm × 2.0 mm, 5 μm, Phenomenex, Torrance, CA, USA) protected by a SecurityGuard C_{18} column (4 mm × 3.0 mm, 5 μm, Phenomenex, Torrance, CA, USA). The column was maintained at room temperature.

The analytes were eluted using a stepwise gradient elution at a flow rate of 0.5 mL/min with mobile phase A consisting of 5 mM ammonium acetate aqueous and mobile phase B consisting of methanol. The gradient program used was as follows: from 0 min to 0.1 min, 5% B; 2.5 min, 40% B; from 2.6 min to 4.5 min, 80% B; and from 4.6 min to 6.0 min, 5% B.

2.4. Mass spectrometric conditions

The mass spectrometer was operated simultaneously in both positive and negative modes. Quantitation was performed by multiple reaction monitoring (MRM). In the positive mode, the MS/MS setting parameters were as follows: 30 psi curtain gas; 45 psi nebulizer gas (GS1); 40 psi turbo gas (GS2); 5500 V ion spray voltage; 400 °C; and 100 ms dwell time. The optimized MRM fragmentation transitions for this mode were *m/z* 428.0 → *m/z* 279.0 for ranolazine, *m/z* 414.0 → *m/z* 265.0 for CVT-2514, *m/z* 248.0 → *m/z* 99.0 for CVT-2738, *m/z* 337.0 → *m/z* 188.0 for fentanyl (IS), and *m/z* 446.0 → *m/z* 321.0 for glipizide (IS). In the negative mode, the MS/MS setting parameters were as follows: the same curtain gas, nebulizer gas and turbo gas as above; −2000 V ion spray voltage; 400 °C; and 100 ms dwell time. The optimized MRM fragmentation transitions were *m/z* 211.0 → *m/z* 123.0 for CVT-4786 and *m/z* 205.0 → *m/z* 171.0 for lipoic acid (IS).

2.5. Preparation of standard and quality control (QC) samples

The standards and QC samples were prepared from two separate stock solutions in parallel. The stock standard solutions of ranolazine (1 mg/mL), CVT-2514 (1 mg/mL), CVT-2738 (1 mg/mL) and CVT-4786 (1 mg/mL) were prepared by dissolving each of the accurately weighted reference compounds in methanol–water (50:50, v/v). A combined standard solution of the metabolites was prepared by adding 50 μL aliquots of each stock solution to a polypropylene tube and made up to 1 mL with methanol–water (50:50, v/v) to obtain the final concentrations of 50 μg/mL of each metabolite. The resulting solutions were then serially diluted with methanol–water (50:50, v/v) to obtain the desired concentrations. The stock standard solutions of IS were prepared by dissolving appropriate amounts of standards in methanol to obtain a final concentration of fentanyl (647 μg/mL), glipizide (502 μg/mL) and lipoic acid (992 μg/mL). The combined IS solutions of 100 ng/mL fentanyl, 100 ng/mL glipizide and 100 ng/mL lipoic acid were prepared by adding 39 μL fentanyl IS stock solution, 50 μL glipizide IS stock solution and 25 μL lipoic acid IS stock solution to a 250 mL volumetric flask, then diluting to the volume with methanol–water (50:50, v/v).

The calibration curves with seven non-zero standard levels contained ranolazine/CVT-2514/CVT-2738/CVT-4786 in the concentration of 4–2000 ng/mL for ranolazine, 4–1000 ng/mL for CVT-2514, 4–1000 ng/mL for CVT-2738, and 8–1000 ng/mL for CVT-4786. These curves were prepared by spiking appropriate volumes of the standard solutions of ranolazine and the metabolites to 100 μL of blank human plasma. The quality control (QC) samples were similarly prepared at three different concentration levels as follows: LQC (12/10/10/24 ng/mL), MQC (100/50/50/100 ng/mL), and HQC (1600/800/800/800 ng/mL), for ranolazine, CVT-2514, CVT-2738, and CVT-4786, respectively. All the solutions were stored at 4 °C and were brought to room temperature before use. The QC samples were aliquoted into 1.5 mL polypropylene tubes and stored at −20 °C until the analysis was performed.

2.6. Sample preparation

The frozen plasma samples were thawed at room temperature and vortexed thoroughly. To a 100 μL aliquot of plasma sample, 100 μL of combined internal standard, 40 μL methanol–water (50:50, v/v) and 600 μL of acetonitrile were added. The mixture was then vortex-mixed for 1 min and then centrifuged at 11,600 × *g* for 5 min. A 50 μL aliquot of the supernatant was transferred into another polypropylene tube with 100 μL water, after vortexing, a 5 μL aliquot of the resulting solution was injected onto the LC–MS/MS system for analysis.

2.7. Method validation

The selectivity of the method was evaluated by analyzing six blank plasma samples and twelve spiked plasma samples at the lower limit of quantification (LLOQ) level from six different sources. The MRM chromatograms of the blank plasma samples were compared with MRM chromatograms obtained when the blank plasma samples from the same source were spiked with the analytes and internal standards. The peak areas of the endogenous compounds co-eluted with the analytes should be less than 20% of the peak area of the LLOQ standard [12].

To evaluate linearity, plasma calibration curves were prepared and assayed in duplication on three consecutive days. The calibration curves (peak area ratios of analyte to IS versus the nominal analyte concentration) were fitted by weighted ($1/x^2$) least squares linear regression.

The accuracy and precision were assessed by the determination of QC samples at three levels (low, medium, and high; Table 1) using six replicates on three validation days. Accuracy was expressed by relative error (RE) and precision by relative standard deviation (RSD). The intra-day and inter-day precisions were required to be below 15%, and the accuracy to be within ±15%.

LLOQ, defined as the lowest concentration at which both precision and accuracy are less than or equal to 20%, was evaluated by analyzing samples which were prepared in six replicates.

The extraction recovery of the analytes was estimated by comparing the peak area ratios of the analytes to IS in the extracted QC samples ($n = 6$) with those of samples to which the corresponding solutions were added post-extraction. The extraction recovery of each IS was determined in a similar way using the QC samples at high concentration as a reference.

Table 1
Precision and accuracy of the assay for determination of ranolazine and three metabolites in human plasma ($n = 6$).

Day analysis		Nominal concentration (ng/mL)											
		Ranolazine			CVT-2514			CVT-2738			CVT-4786		
		12.0	100	1600	10.0	50.0	800	10.0	50.0	800	24.0	100	800
1	Within-day mean	12.5	104	1410	10.5	54.1	766	10.0	50.8	775	22.8	98.9	795
	S.D.	0.5	3.8	22	0.2	1.3	35	0.2	1.3	31	1.0	6.4	32
	R.S.D. (%)	4.0	3.6	1.6	1.8	2.5	4.5	2.4	2.6	4.0	4.3	6.4	4.0
	Relative error (%)	3.8	3.9	−11.9	5.3	8.1	−4.3	−0.4	1.5	−3.2	−4.8	−1.1	−0.6
2	Within-day mean	13.1	110	1540	11.4	57.1	834	10.2	49.3	794	25.6	103	848
	S.D.	0.7	5.5	55	0.4	0.8	24	0.5	1.7	35	0.8	3.2	32
	R.S.D. (%)	5.1	5.0	3.6	3.6	1.4	2.9	5.3	3.4	4.4	3.2	3.2	3.8
	Relative error (%)	9.4	10.7	−3.8	13.9	14.2	4.3	2.5	−1.4	−0.8	6.6	2.9	6.0
3	Within-day mean	12.2	98.4	1434	10.8	52.8	794	10.2	50.0	722	26.7	107	836
	S.D.	0.3	7.0	26	0.6	2.4	16	0.6	3.1	19	1.4	9.0	16
	R.S.D. (%)	2.7	7.1	1.8	5.5	4.6	2.1	5.5	6.2	2.6	5.2	8.5	2.0
	Relative error (%)	1.6	−1.6	−10.4	8.1	5.7	−0.7	2.5	0.0	−9.8	11.4	6.6	4.5
	Overall mean	12.6	104	1461	10.9	54.7	798	10.2	50.0	763	25.1	103	826
	S.D.	0.6	7	68	0.5	2.4	38	0.5	2.1	42	2.0	7.0	35
	R.S.D. (%)	5.1	7.0	4.6	5.0	4.4	4.7	4.6	4.2	5.5	7.8	6.8	4.2
	Relative error (%)	4.9	4.3	−8.7	9.1	9.3	−0.2	1.5	0.0	−4.6	4.4	2.8	3.3

Blank plasma from six different sources were extracted and then spiked with analytes and internal standards to assess the matrix effect (ME). The corresponding peak area ratios of the analytes to IS in spiked plasma post-extraction (A) were then compared with those of the water-substituted samples (B) at equivalent concentrations. The ratio ($A/B \times 100$) is defined as the ME.

All four analytes were tested with regard to long-term and short-term stabilities by analyzing plasma samples at low (12/10/10/24 ng/mL for ranolazine/CVT-2514/CVT-2738/CVT-4786) and high (1600/800/800/800 ng/mL for ranolazine/CVT-2514/CVT-2738/CVT-4786) concentrations. The long-term stability was evaluated after storage of the samples at −40 °C for 7 days and −20 °C for 60 days. The short-term stability was assessed after the exposure of the spiked samples at room temperature for 6 h, the ready-to-inject samples (after extraction) in the LC autosampler at 4 °C for at least 24 h, and the freeze/thaw cycles on consecutive days. The criterion used for all the stability measurements was 85–115% for mean accuracy. Additionally, the stabilities of the analytes and IS stock solutions at 4 °C for 59 days to 74 days were performed.

2.8. Assay application to a clinical study

The validated LC–MS/MS assay was applied to investigate the plasma profiles of ranolazine and its three metabolites. Single doses of 500 and 1500 mg ranolazine extended-release tablets (Fujian Fukang Pharmaceutical Co., Ltd., Fujian, China) were orally administered to 20 Chinese healthy volunteers. The pharmocokinetic study was approved by the Ethics Committee. The blood samples (4 mL) were collected into sodium heparin containing tubes before and 0.5, 1, 1.5, 2, 2.5, 3, 3.5, 4, 4.5, 5, 5.5, 6, 8, 12, 24 and 36 h after dosing, and centrifuged at 3164 *g* for 10 min to separate the plasma fractions. The plasma samples were stored at −40 °C (less than 24 h) and then transferred to −20 °C until analysis.

3. Results and discussion

3.1. Mass spectrometry

During the early stage of method development, using ESI source or APCI source was investigated. It was found that ESI could offer much higher signal intensity for the analytes than APCI. Consequently, ESI was chosen as the ionization source in the present experiment. Mass spectrometer was operated in both positive and

Table 2
Stability of ranolazine and the metabolites in human plasma ($n = 3$).

	Concentration (ng/mL)		R.S.D. (%)	R.E. (%)
	Added	Found (mean)		
Benchtop stability (room temperature for 6 h)				
Ranolazine	12.0	13.1	5.2	8.9
	1600	1468	1.3	−8.3
CVT-2514	10.0	10.7	3.7	7.2
	800	789	1.4	−1.4
CVT-2738	10.0	11.4	5.4	14.3
	800	791	5.2	−1.2
CVT-4786	24.0	24.8	6.9	3.3
	800	826	1.1	3.2
Three freeze–thaw cycles				
Ranolazine	12.0	11.1	10.5	−7.1
	1600	1548	0.9	−3.2
CVT-2514	10.0	11.1	4.2	10.9
	800	879	0.9	9.9
CVT-2738	10.0	10.2	5.8	1.9
	800	731	1.0	−8.6
CVT-4786	24.0	24.6	2.3	2.5
	800	787	1.9	−1.6
Processed-sample stability (4 °C for 24 h)				
Ranolazine	12.0	12.6	6.1	4.7
	1600	1373	0.8	−14.2
CVT-2514	10.0	10.5	1.5	4.8
	800	736	2.2	−8.0
CVT-2738	10.0	10.5	7.9	5.0
	800	743	3.4	−7.2
CVT-4786	24.0	26.2	4.4	9.3
	800	882	1.4	10.3
Frozen matrix stability (−40 °C for 7 days)				
Ranolazine	12.0	13.3	3.3	10.8
	1600	1647	0.9	2.9
CVT-2514	10.0	10.6	7.5	6.1
	800	772	0.4	−3.5
CVT-2738	10.0	10.4	8.4	3.5
	800	761	2.3	−4.8
CVT-4786	24.0	23.5	2.6	−2.1
	800	786	2.0	−1.7
Frozen matrix stability (−20 °C for 60 days)				
Ranolazine	12.0	12.4	11.7	3.2
	1600	1499	2.1	−6.3
CVT-2514	10.0	10.2	4.7	2.4
	800	759	5.0	−5.1
CVT-2738	10.0	9.4	5.9	−5.6
	800	774	1.8	−3.2
CVT-4786	24.0	21.1	4.5	−12.3
	800	734	1.6	−8.3

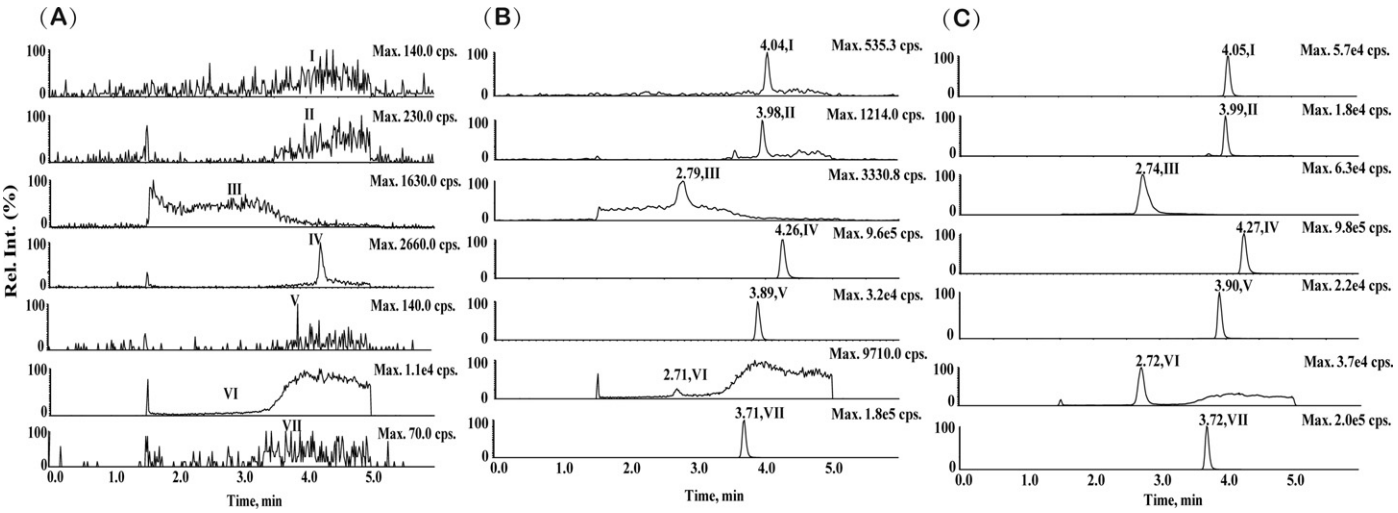

Fig. 2. Typical chromatograms for ranolazine, three metabolites, and their IS in human plasma. (A) Blank plasma sample, (B) plasma sample spiked with 4 ng/mL ranolazine, 4 ng/mL CVT-2514, 4 ng/mL CVT-2738, 4 ng/mL CVT-4786 and 100 ng/mL combined IS; (C) plasma sample 5.5 h after an oral administration of 500 mg ranolazine extended-release tablet (measured concentration of ranolazine, CVT-2514, CVT-2738, and CVT-4786 is 596, 67.0, 111, and 151 ng/mL, respectively). Peaks I–VII refer to ranolazine, CVT-2514, CVT-2738, fentanyl (IS), glipizide (IS), CVT-4786, and lipoic acid (IS), respectively.

negative modes. The positive ionization mode was selected for the quantification of ranolazine, CVT-2514 and CVT-2738 because of the presence of a piperazine group. The negative ionization mode was selected owing to the presence of a carboxylic acid group for CVT-4786. In the positive ion mode, ranolazine, CVT-2514, CVT-2738, and their IS, fentanyl and glipizide, formed protonated molecules $[M+H]^+$ at m/z 428.0, 414.0, 248.0, 337.0, and 446.0, respectively, as the base peak ions in full-scan Q1 mass spectra. Moreover, in the negative mode, the ions with m/z 211.0 and 205.0 as deprotonated molecules $[M-H]^-$ represented CVT-4786 and lipoic acid, respectively. For MRM mass spectrometric detection, the most prominent ions produced by the analytes and their IS were selected as described in Section 2.4. Fig. 1 shows the product ion mass spectra.

3.2. Chromatography

A major challenge encountered in the simultaneous determination of their concentrations was to achieve sufficient retention because of the different polarities of the four analytes. To solve this problem, three C_{18} columns (Xbridge C_{18}, Capcell C_{18} and Gemini C_{18}) were evaluated in terms of sensitivity, peak shape, baseline noise and retention time. The result showed that the Gemini C_{18} column performed better sensitivity and retention of the analytes than the other two kinds, thus, it was chosen for further evaluation. Second, CVT-2738 and CVT-4786 are relatively more polar than the other two analytes, so isocratic elution could not meet the requirement of the simultaneous detection. An appropriate gradient elution condition was established to optimize the chromatographic behavior, as well as eliminate interference peaks.

In addition, methanol and acetonitrile as the organic mobile phase were investigated to obtain good retention time and sensitivity. The results showed that methanol as mobile phase could provide higher intensity. Ammonium acetate as a buffer in the mobile phase is important to maintain good peak shape and reproducibility of retention time over hundreds of injections. This characteristic of ammonium acetate ensured the ruggedness of this method. Thus, the mobile phase was finally optimized as methanol: 5 mM ammonium acetate using a gradient elution.

3.3. Method validation

3.3.1. Selectivity

Selectivity was evaluated by extracting blank human plasma from six different lots of matrices and comparing the MS/MS response at the retention times of ranolazine and three metabolites to the responses of the LLOQ. Fig. 2 shows the typical chromatograms of a blank plasma sample, a blank plasma sample spiked with ranolazine and the metabolites at the LLOQ and IS, and a plasma sample from a healthy volunteer after an oral administration. No significant interferences from endogenous substances was observed in any of the blank plasma samples for the analytes. Besides, the selectivity of each analyte when combined determination with the others was estimated by adding one analyte's solution at the upper limit of quantitation level to the blank and checking the other three ion channels if there was interference. The results showed that no interference was found.

3.3.2. Linearity of calibration curves and lower limits of quantification (LLOQ)

The linear regressions of the peak area ratios versus concentrations were fitted over the concentration range of 4–2000 ng/mL for ranolazine, 4–1000 ng/mL for CVT-2514 and CVT-2738, 8–1000 ng/mL for CVT-4786 in human plasma. The typical equations of the calibration curve are as follows:

Ranolazine: $y = 7.69e^{-5}x + 6.42e^{-5}$, $r = 0.9960$
CVT-2514: $y = 1.70e^{-4}x + 2.47e^{-4}$, $r = 0.9977$
CVT-2738: $y = 2.96e^{-2}x + 3.86e^{-3}$, $r = 0.9984$
CVT-4786: $y = 2.52e^{-3}x - 2.24e^{-3}$, $r = 0.9977$

where y represents the ratio of analyte peak area to that of IS, and x represents the plasma concentration.

The LLOQ was 4/4/4/8 ng/mL for ranolazine, CVT-2514, CVT-2738, and CVT-4786. These limits are sufficient for the clinical pharmacokinetic study of ranolazine and its three metabolites following an oral administration of ranolazine [6]. The precision at LLOQ of the four analytes was between 5.5% and 12.0%, and the accuracy was between −3.4% and 4.7%.

Table 3
Comparison of bioanalytical methods for ranolazine.

Compound(s)	Sample preparation/ sample size (mL)	Internal standard(s)	Biological matrix	Detection technique	LLOQ (ng/mL)	Analytical run time (min)	References
Ranolazine	LLE/1	Resperidone	Human plasma	HPLC	200	6	[7]
Ranolazine	LLE/0.1	Phenoprolamine	Human plasma	LC–MS/MS	5	2	[5]
Ranolazine	Protein precipitation/0.9	Aripiprazole	Human plasma	LC–MS/MS	5	10	[6]
Ranolazine	LLE/0.02	Phenoprolamine	Rat plasma	LC–MS	20	4.2	[8]
Ranolazine	LLE	Tramadol	Human plasma	LC–MS/MS	10	NA	[9]
Ranolazine metabolites (semiquantitation)	SPE/1	RS-87986	Human plasma	LC–MS	20	>5	[11]
Ranolazine	Protein precipitation/0.1	Fentanyl, glipizide, lipoic acid	Human plasma	LC–MS/MS	4	6	This article
CVT-2514					4		
CVT-2738					4		
CVT-4786					8		

3.3.3. Precision and accuracy

Eighteen replicates of the QC samples from three consecutive runs were used to evaluate precision and accuracy at each concentration level. The intra-assay precision for all compounds was less than 8.5%, and the inter-assay precision was less than 7.8%. The inter-assay mean accuracy was between −8.7% and 9.3% (Table 1).

3.3.4. Matrix effect

ME is a primary issue when a new LC–MS/MS method is established especially using a Turbo Ionspray ionization interface. MEs occur when molecules co-eluting with the compounds of interest alter the ionization efficiency of the electrospray interface. The importance of MEs on the reliability of HPLC–ESI–MS/MS has been shown in terms of accuracy and precision [13]. In the present study, the evaluation of the ME was conducted in six different batches of human plasma following the procedures described above. The MEs for all compounds ranged from 92.7% to 107%. Therefore, ion suppression or enhancement from the plasma matrix was negligible under the current conditions.

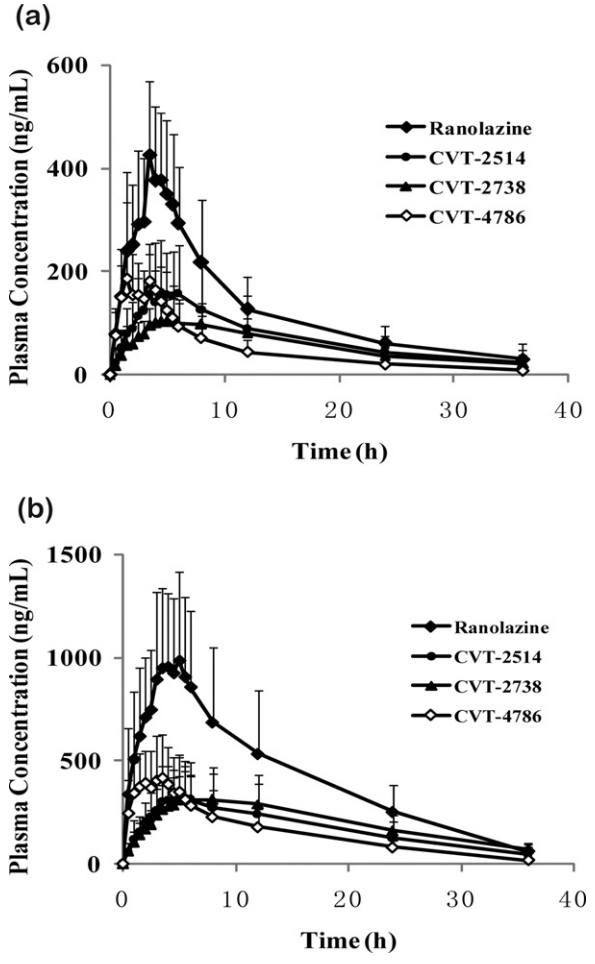

Fig. 3. Mean plasma concentrations of ranolazine after a single dose of oral administration of (a) 500 mg and (b) 1500 mg ranolazine extended-release tablets to 10 healthy volunteers of each dose level.

3.3.5. Extraction recovery

The mean extraction recoveries of four analytes and internal standards were between 84.2% and 108%.

3.3.6. Stability

The stability tests of the samples subjected to exposure at room temperature at least for 6 h, storage at −40 °C for 7 days, storage at −20 °C for 60 days, three freeze–thaw cycles and storage in the autosampler at 4 °C after extraction for at least 24 h were studied at two concentration levels (low and high QC) in three replicates. The results showed that each analyte had an acceptable stability under the test conditions (Table 2).

The stability of stock solutions of ranolazine, CVT-2514, CVT-2738, CVT-4786, fentanyl, glipizide, and lipoic acid used in the preparation of standards was established at 4 °C for 59 days to 74 days as part of the validation. Results for the determination of stock solution stability were calculated by comparing the mean peak area of stability solutions to that of freshly prepared control solutions. In addition, the room temperature stability of all seven standard working solutions was established for at least 6 h. Results showed that the difference between the stored solutions was within ±6%, thereby indicating acceptable stability for these durations of storage.

3.3.7. Advantages compared with methods published previously

The main advantage of this method is the simultaneous determination of ranolazine and its three metabolites. Furthermore, this assay have several advantages compared with bioanalytical methods for ranolazine published previously, such as high sensitivity (the LLOQ of ranolzine is 4 ng/mL), short analytical run time (6 min for four analytes and three internal standards), and simple preparation procedure (protein precipitation) with a small sample volume (100 μL). Additional details are summarized in Table 3.

3.4. Application of the method to a pharmacokinetic study in healthy volunteers

The assay described in the present research was applied to study the pharmacokinetic profiles of ranolazine and its three metabolites in Chinese healthy volunteers. The mean plasma concentration-time profiles of ranolazine and the metabolites after

a single dose of oral administration of 500 and 1500 mg ranolazine extended-release tablets are shown in Fig. 3. From the results of our study, the ratios of AUC_{0-t} to $AUC_{0-\infty}$ in most subjects were more than 80%. This finding indicates that the sampling time to 36 h is sufficient.

4. Conclusion

A sensitive bioanalytical method was successfully developed and validated for the simultaneous determination of ranolazine and its three metabolites, CVT-2514, CVT-2738 and CVT-4786 in human plasma. The linear range of the method was 4–2000, 4–1000, 4–1000, and 8–1000 ng/mL for ranolazine, CVT-2514, CVT-2738, and CVT-4786, respectively.

The main advantage of this assay is its efficiency in analyzing the four analytes simultaneously. The success of this validated method allowed for its application in the pharmacokinetic study, thus providing an efficient support for further clinical studies.

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