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Determination of mildronate by LC–MS/MS and its application to a pharmacokinetic study in healthy Chinese volunteers

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ABSTRACT

A simple, rapid and accurate liquid chromatography–tandem mass spectrometry (LC–MS/MS) method has been developed and validated for the determination of mildronate in human plasma. Following a simple protein precipitation with methanol, the analyte was separated on a C_{18} column by isocratic elution with methanol and 10 mM ammonium acetate (55:45; v/v), and then analyzed by mass spectrometry in the positive ion MRM mode. Good linearity was achieved over a wide range of 0.01–20 μg/mL. The intra- and inter-batch precisions (as RSD, %) were less than 7.1%. The average extraction recovery was 87.5%. The method described above has been used, for the first time, to reveal the pharmacokinetics of mildronate injection in healthy subjects. After single intravenously administration of 250, 500 and 1000 mg mildronate, the elimination half-life ($t_{1/2}$) were (5.56 ± 1.55), (6.46 ± 1.07) and (6.55 ± 1.17) h, respectively. The Student–Newman–Keuls test results showed that peak plasma concentration (C_{max}) and the area under the plasma concentration versus time curve from time 0 to 24 h (AUC_{0-24}) were both linearly related to dose. The pharmacokinetics of mildronate fitted the linear dynamic feature over the dose range studied. The essential pharmacokinetic parameters of multidoses administration intravenously (500 mg, b.i.d) were as follows: $t_{1/2}$ was (15.34 ± 3.14) h; C_{max} was (25.50 ± 3.63) μg/mL; AUC_{0-24} was (58.56 ± 5.57) mg h/L. The $t_{1/2}$ and AUC of multidoses administration intravenously were different from those of single-dose administration significantly. These findings suggested that accumulation of mildronate in plasma occurred.

1. Introduction

Mildronate [3-(2,2,2-trimethylhydrazinium) propionate dihydrate, inner salt] is an analogue of carnitine [1]. The chemical structure of mildronate is shown in Fig. 1A. It is a new type of medicine for heart protection which can lower the intracellular concentration of free carnitine and thus suppress fatty acid oxidation and facilitate glycolysis during ischemia [2,3]. It was developed by Latvia Organic Synthetic Inst and was put into market in the Russian Federation in 2002. Recently, the formulation of mildronate injection has been developed and approved to conduct clinical trial in China. To date, there has been no report studying the pharmacokinetic characteristics of mildronate in man. Therefore, it is urgent to investigate the pharmacokinetic properties of mildronate in man. The aim of our study was to assess the pharmacokinetic properties of mildronate in healthy Chinese volunteers.

As mildronate's structure is simple and has no visible absorption peak in UV district and fluorescence, ultraviolet or fluorescence detectors cannot be used for the detection [4]. At the same time, because of the strong polarity, mildronate is hard to be separated from the endogenous substances in plasma by HPLC system without derivatization or radioisotopic exchange [5,6]. Thus, HPLC–MS/MS analytical procedure is developed for the determination of mildronate with good specificity and reproducibility. So far as we know, only one LC–MS/MS method for the determination of mildronate in plasma and urine has been published recently [7]. However, the analytical method for mildronate was not applied in the pharmacokinetic study. We developed a method with simpler sample preparation procedures, shorter analytical time and lower labor-costing in comparison with the reported method, which was efficient in analyzing large numbers of plasma samples obtained from pharmacokinetic studies after intravenous infusion of mildronate injection. The pharmacokinetic profiles of mildronate injection were investigated for the first time in man.

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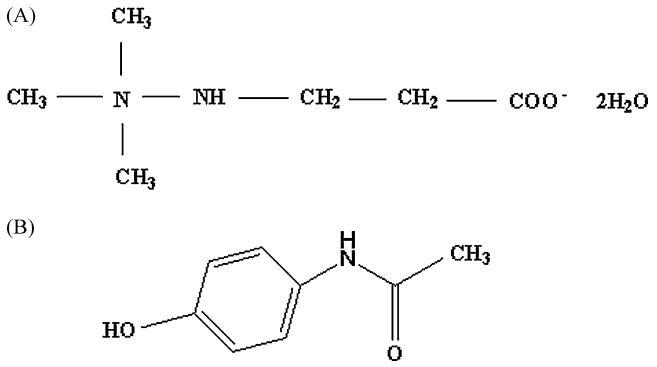

Fig. 1. The chemical structure of mildronate (A) and acetaminophen (B).

2. Experimental

2.1. Reagent and materials

Mildronate injection was obtained from Shengtai Pharmaceutical Co., Ltd. (Shanxi, China). Mildronate reference standard (99.1% purity) was provided by Jinan Lanhai Chemical Limited Company (Shandong, China). Acetaminophen, the internal standard (IS), was purchased from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). Methanol of HPLC-grade was purchased from Fisher Scientific (Pittsburgh, PA, USA). Water was distilled and purified using a Milli-Q Water Purification System (Millipore, Bedford, MA, USA). Other chemicals were all of analytical grade. Drug-free human plasma from healthy volunteers was kindly provided by the Blood Center of Xijing Hospital (Shaanxi, China) and was stored at −20 °C.

2.2. Instrumentation

The LC–MS/MS procedure was performed using an Agilent 1200 series HPLC and an Agilent 6410 Triple Quadrupole mass spectrometer equipped with an electrospray ionization source (Agilent Technologies, USA). The chromatographic separation was achieved on a Shim-pack VP-ODS C_{18} (150 mm × 4.6 mm, 5 μm) column. All data were acquired employing Agilent 6410 Quantitative Analysis version analyst data processing software.

2.3. Chromatographic conditions

The mobile phase was a mixture of methanol–10 mM ammonium acetate solution (55:45; v/v), which was pumped at a flow rate of 0.4 mL/min. The column oven temperature was set at 35 °C. The total run time was 5.8 min. The sample injection volume was 10.0 μL. Mass spectrometric detection was performed on a Series 6410 Triple Quad LC–MS/MS (Agilent Technologies, USA) in electrospray positive ionization using multiple reaction monitoring (MRM). The mass transition was m/z 147.2 → 58.3 for mildronate, and m/z 152.0 → 110.0 for IS, respectively. The other working parameters of the mass spectrometer were as follows: dwell time 200 ms; gas flow 10 L/min; gas temperature 350 °C; nebulizer pressure 50 psi; fragmentor voltage 60 V (mildronate) and 90 V (IS), collision energy 36 eV (mildronate) and 14 eV (IS).

2.4. Sample preparation

The 200 μL aliquot of plasma sample was mixed with 50 μL IS (10 μg/mL) and 20 μL methanol in a 1.5 mL tube. The mixture was vortexed for 30 s, precipitated with 700 μL methanol, vortexed for 3 min, and then centrifuged at 10,000 × *g* for 10 min. 10 μL aliquot of the supernatant was injected into the HPLC–MS/MS system for analysis.

2.5. Preparation of the stock and standard solutions

The stock solutions of mildronate (1 mg/mL) and the internal standard (100 μg/mL) were separately prepared with methanol. Standard solutions of mildronate at concentrations of 100 μg/mL, 50 μg/mL, 25 μg/mL, 10 μg/mL, 5 μg/mL, 2 μg/mL, 1 μg/mL, 500 ng/mL, 250 ng/mL, 200 ng/mL and 100 ng/mL were prepared by serial dilution of mildronate stock solution with methanol. A solution containing 10 μg/mL of acetaminophen was also obtained by dilution of the internal standard stock solution with methanol. All standard solutions were kept at −20 °C.

2.6. Preparation of calibration curves and quality control samples

The calibration curves of mildronate were prepared at the concentration levels of 0.01, 0.025, 0.05, 0.1, 0.2, 0.5, 1, 5, 10 and 20 μg/mL by spiking 200 μL blank human plasma with appropriate amount of the standard solution. Three levels of mildronate quality control samples were prepared in blank human plasma at the nominal concentrations of 0.02, 2.5 and 10 μg/mL. All samples were stored at −20 °C until analysis.

2.7. Method validation

A thorough and complete method validation of mildronate determination in human plasma was done following the USFDA guidelines [8]. The method was validated for specificity, sensitivity, linearity, accuracy and precision, recovery, matrix effect and stability.

The specificity of the method was checked by comparing the chromatograms of six different batches of blank human plasma with the corresponding spiked plasma. Each blank plasma sample was tested using the precipitation procedure and chromatographic conditions described above to ensure no interference of mildronate and IS from plasma.

The linearity was assessed by assaying calibration curves in human plasma on three consecutive batches. And the curves were fitted by a weighted ($1/x^2$) least-squares linear regression method through the measurement of the peak area ratio of the analyte to IS. The acceptance criterion for a calibration curve was a correlation coefficient (r) of 0.99 or better, and that each back-calculated standard concentration must be within 15% deviation from the nominal value except at the LLOQ, for which the maximum acceptable deviation was set at 20%. The LLOQ was defined as the lowest concentration in the standard curve at which the relative standard deviation (RSD) ($n = 6$) was within 20% and relative error (RE) was within ±20%.

The accuracy and precision of this analytical method were evaluated using QC samples. QC samples were prepared as described above. The QC samples were analyzed five times a day to evaluate intra-day precision. The same procedure was performed once a day for three consecutive days to determine inter-day precision. The precision was calculated by using the RSD and a one-way analysis of variance (ANOVA). Accuracy was determined by comparing the calculated concentration using calibration curves with known concentration. The accuracy was expressed as RE.

Recovery presents the extraction efficiency of a method, which was performed at three QC levels (five samples each). The recoveries were evaluated by comparing peak areas of analytes in spiked plasma samples with those of samples to which the analytes had been added after extraction.

The matrix effect was defined as the direct or indirect alteration or interference in response due to the presence of unintended or

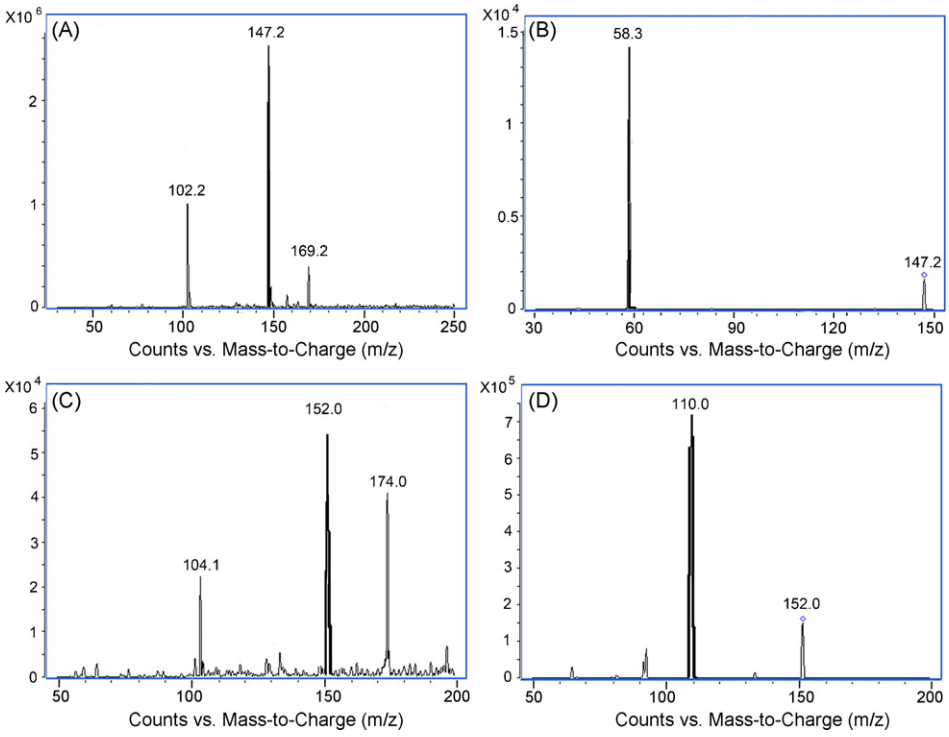

Fig. 2. Full-scan positive product ion mass spectra of (A) precursor ion spectrum of mildronate; (B) product ion spectrum of mildronate; (C) precursor ion spectrum of IS and (D) product ion spectrum of IS.

other interfering substances in the samples. It was evaluated by comparing the peak area of the analytes dissolved in the blank plasma sample's precipitated solution with that of the analytes dissolved in the mobile phase. Three different concentration levels of mildronate (0.02, 2.5 and 10 μg/mL) were evaluated by analyzing five samples at each level. The blank plasma used in this study was from five different batches of the blank plasma. If the peak area ratio is less than 85% or more than 115%, a matrix effect is implied.

Stability experiments were performed to evaluate the stability of the analyte in plasma under different conditions. QC samples were subjected to short-term room temperature condition for 24 h, to long-term storage conditions (−20 °C) for 2 months, to post-preparative stability of the processed samples after 24 h and to three freeze–thaw stability studies. All stability studies were conducted at three QC levels (0.02, 2.5 and 10 μg/mL as low, middle and high values) with five determinations for each.

2.8. Application for pharmacokinetic study

The pharmacokinetics of mildronate injection was studied in healthy Chinese subjects in accordance with the Declaration of Helsinki for biomedical research involving human subjects and Good Clinical Practice. The protocol and associated informed consent statements were reviewed and approved by the Committee on Human Rights Related to Human Experimentation at Xijing Hospital and the informed consent statements were signed by the volunteers.

Thirty healthy male and female subjects (aged 29–39, body weight 55–68 kg) were enrolled in this study. They had no history of cardiovascular, hepatic, renal, gastrointestinal, hematologic, and nervous or any acute or chronic diseases or drug allergy, and had stopped using any drugs 2 weeks before the study. Physical examination and laboratory tests showed no abnormal findings. All subjects were randomly divided into three groups, such as Groups A–C (five males and five females in each group). Groups A–C were administered a single dose of 250, 500 and 1000 mg mildronate injection which were diluted in 100 mL 5% glucose, respectively. After overnight fasting, each subject was administered with the solution via intravenous infusion for 1 h. Water intake was allowed 2 h post-dose and standard meals were provided at 4 and 10 h post-dose. The subjects were required to refrain from smoking, alcohol, caffeine and strenuous exercise during the study and were under direct medical supervision at the study site. Blood samples (4 mL) were collected at 0 h (pre-dose) and 0.25, 0.5, 0.75, 1, 1.5, 2, 3, 4, 6, 8, 12 and 24 h post-dose. The samples were transferred to heparinized tube and centrifuged at 3000 × *g* for 10 min. Plasma was separated and stored at −80 °C until analysis. In the design of multidoses, Group B received 500 mg of mildronate injection at 8:00 a.m. and 8:00 p.m. for consecutive 6 days. At days 3, 4 and 5, 4 mL of venous blood was drawn to observe minimum value of steady plasma-drug concentration before every dosing at 8:00 a.m. At day 7, the procedure was the same as that of single dose mentioned above. The plasma mildronate concentrations were determined by LC–MS/MS method developed in this study.

The plasma concentrations of mildronate versus time profiles were acquired for each subject. The peak plasma concentration (C_{max}) and the time to C_{max} (T_{max}) were obtained directly from the data. The other major pharmacokinetic parameters were calculated by non-compartmental method. The elimination rate constant (k) was estimated from the least-squares regression slope of the terminal portion of the plasma concentration–time curve. The apparent elimination half-life ($t_{1/2}$) was calculated as $\ln 2/k$. The area under the plasma concentration–time curve from time zero (pre-dose) to the last measurable concentration (AUC_{0-24}) was calculated using the linear trapezoidal rule. The AUC from 0 to infinity ($AUC_{0-\infty}$) was calculated as the $AUC_{0-\infty} = AUC_{0-t} + C_t/k$. C_t is the last plasma concentration evaluated in plasma greater than the LLOQ. The mean residence time (MRT) was obtained by dividing the area under the first moment–time curve ($AUMC_{0-\infty}$) by the area under the curve ($AUC_{0-\infty}$). Vd/F was the apparent volume of distribution. CL/F was the clearance rate. C_{ssmin} was minimum steady-state plasma concentration. C_{av} was the average steady-state plasma concentra-

tion and AUC_{ss} was the area under the concentration–time curve for steady-state conditions. The data analysis of pharmacokinetic parameters was performed by using Drug and Statistic software (Version 2.1.1, Chinese).

3. Results and discussion

3.1. Method development

3.1.1. Selection of IS

It was difficult to find a compound that could ideally mirror the analyte to serve as a suitable IS. Several compounds were investigated such as dimethylbiguanide, and phenethylbiguanide, but they had poor peak shape under the condition and finally acetaminophen (Fig. 1B) was found to be the most appropriate for the present purpose. The behavior of acetaminophen's retention time was similar to that of mildronate. Chromatograms were obtained and no significant direct interference in the MRM channels at the relevant retention times was observed.

3.1.2. Sample pre-treatment

In this method, the plasma sample was precipitated with methanol and then the extracts were injected into the mobile phase stream without evaporation and reconstitution. It could simplify the sample preparation procedure significantly and also meet the requirements of the assay. No interference was observed from any endogenous or exogenous plasma matrix.

3.1.3. Liquid chromatography

Chromatographic analysis of the analyte and IS was initiated under isocratic conditions with an aim to develop a simple separation process with a short run time. Feasibility of various mixture(s) of solvents such as acetonitrile and methanol using different buffers such as ammonium acetate, ammonium formate and formic acid, along with altered flow rates (in the range of 0.2–0.6 mL/min) were tested to identify the optimal mobile phase that produced the best sensitivity, efficiency and peak shape. It was found that a mixture of methanol–10 mM ammonium acetate (55:45; v/v) could achieve this purpose and was finally used as the mobile phase. A flow rate of 0.4 mL/min permitted a run time of 5.8 min.

3.1.4. Mass spectrometry

In order to develop ESI conditions for mildronate and IS, quadrupole full scans were carried out in positive ion detection mode. The solutions containing mildronate and IS were injected directly into the mass spectrometer respectively. Under these conditions, the analytes yielded major $[M+H]^+$ ions at m/z 147.2 for mildronate and m/z 152.0 for IS. Each of the precursor ions was subjected to collision-induced dissociation to determine the resulting product ion. The product ion mass spectra of mildronate and the IS are shown in Fig. 2. The results showed that the most sensitive mass transition were m/z 147.2 → 58.3 for mildronate and m/z 152.0 → 110.0 for IS. The MRM state file parameters were the optimized values for the sensitivity and specificity required for mildronate.

3.2. Specificity and sensitivity (LLOQ)

The specificity of the method towards endogenous plasma matrix was evaluated in six different batches of human plasma. Fig. 3 shows the typical chromatograms of blank plasma, spiked plasma with mildronate and the IS, and the plasma from a volunteer after administration. The retention times of mildronate and IS were 4.1 and 5.0 min, respectively. No significant direct interference in the blank plasma traces was observed from endogenous substances

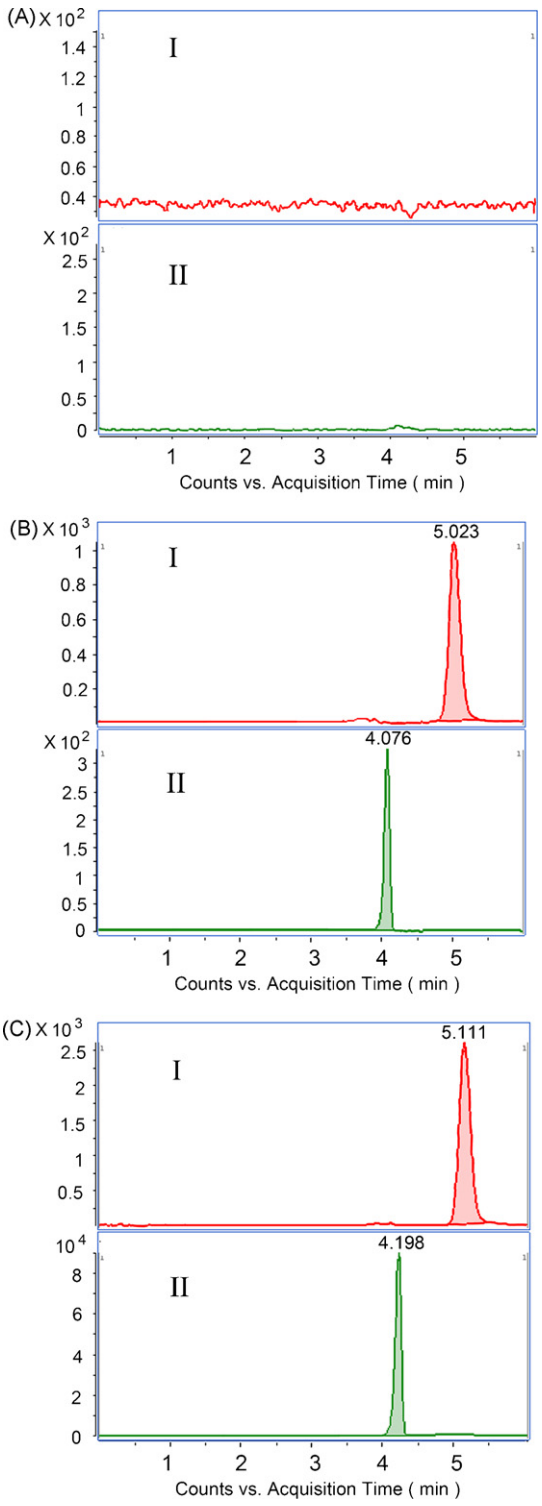

Fig. 3. Typical chromatograms of mildronate and IS in human plasma: (A) blank plasma sample; (B) a blank plasma sample spiked with mildronate (0.02 μg/mL) and IS; and (C) a plasma sample obtained from a volunteer at 2 h after intravenous infusion of 250 mg mildronate. (I: IS, IL: mildronate).

in drug-free human plasma at the retention time of mildronate or the IS.

In our pilot study, the lowest plasma concentrations of the 250 mg group exceeded 0.02 μg/mL. In order to obtain the good linearity and avoid instrument carry-over resulting from high concentration, we defined the linear range as 0.01–20 μg/mL. Samples exceeding 20 μg/mL were diluted by the same blank plasma

Table 1
Intra- and inter-batch precision and accuracy for determination of mildronate in human plasma.

Spiked (μg/mL)	Intra-batch precision and accuracy ($n = 5$)			Inter-batch precision and accuracy ($n = 3$)		
	Measured (mean ± S.D.) (μg/mL)	RSD (%)	RE (%)	Measured (mean ± S.D.) (μg/mL)	RSD (%)	RE (%)
0.02	0.020 ± 0.002	5.2	−0.4	0.020 ± 0.001	6.9	−1.6
2.50	2.59 ± 0.17	6.5	1.6	2.67 ± 0.15	5.7	4.7
10.00	10.41 ± 0.80	7.6	2.1	10.71 ± 0.76	7.1	5.0

Table 2
Short-term, post-preparative, and freeze–thaw stability of mildronate in human plasma.

Spiked concentration of mildronate (μg/mL)	Mean of percentage remaining (%)		
	0.02	2.5	10
Short-term stability (24 h, room temperature, $n = 5$)	108.1	106.9	101.2
Post-preparative stability (24 h, room temperature, $n = 5$)	96.4	97.9	97.9
Freeze and thaw stability (three cycles, −20 °C/room temperature, $n = 5$)	101.7	106.7	96.3
Long-term stability (60 d, −20 °C, $n = 5$)	104.6	102.5	107.1

before pre-treatment. The LLOQ of mildronate was established at 0.01 μg/mL, which was sensitive enough for pharmacokinetic study of mildronate injection in human. The precision and accuracy at this concentration was acceptable, with 9.1% of the RSD and 0.7% of the RE ($n = 6$).

3.3. Linearity, accuracy and precision

The calibration curves were constructed by plotting the peak area ratios (y) of mildronate to IS versus the plasma concentrations (x) of mildronate. Good linearity was obtained over the concentration range of 0.01–20 μg/mL with correlation coefficients $r = 0.995$. A typical equation of the calibration curve was $f = 0.027 + 3.067 \times C$ ($r^2 = 0.998$) for mildronate, where f represents the mildronate peak area to the IS peak area ratio and C represents the mildronate concentration. A weighing factor of 1/square of concentration ($1/x^2$) was chosen to achieve homogeneity of variance.

The intra- and inter-batch precision and accuracy of the assay were assessed by analyzing QC samples. The precision was calculated using one-way ANOVA. The results, summarized in Table 1, demonstrate that the precision and accuracy values are within the acceptable range and the method is accurate and precise.

3.4. Recovery and matrix effect

The extraction recovery of mildronate was calculated by analyzing five replicates at 0.02, 2.5 and 10 μg/mL. The extraction recoveries of the assay were 83.6 ± 5.1, 89.7 ± 5.0 and 89.3 ± 3.6% for the low, middle and high concentrations, respectively.

To study the effect of matrix on analyte quantification with respect to consistency in signal suppression, matrix effect was checked with three different batches of heparinized plasma. Three replicates for each of three concentrations (0.02, 2.5 and 10 μg/mL) were prepared from different batches of plasma. The results were 95.3 ± 4.5, 93.6 ± 2.4 and 98.0 ± 2.0 for the low, middle and high concentrations, respectively. In this study, there was no matrix effect of the analytes.

3.5. Stability

The stability results are summarized in Table 2. The data indicated that mildronate was stable under the conditions evaluated, reflecting actual sample handling and analysis. Stability of the QC samples after 24 h at room temperature and after the three freeze and thaw cycles, and post-preparative stability of the processed samples after 24 h were acceptable. Mildronate in plasma stored at −20 °C was stable for 2 months. The RSD of the standard solution long-term freezer stability for 30 days using the concentration at 1 μg/mL was 2.8%. This was coincided with what was reported previously [7].

3.6. Pharmacokinetic studies

No pharmacokinetic study of mildronate injection has been reported so far as we know. The developed method in this paper was successfully used for a pharmacokinetic study in which plasma concentration of mildronate up to 24 h after intravenous infusion of 250, 500 and 1000 mg in 30 healthy Chinese volunteers (Groups A–C) were determined. Mean plasma concentration–time profiles are presented in Fig. 4. By using Drug and Statistics software (Version 2.1.1, Chinese) analysis, the non-compartmental pharmacokinetic parameters are listed in Table 3. The Student–Newman–Keuls test results showed that C_{max} and AUC_{0-24} were both linearly related to the doses. The pharmacokinetics of mildronate fitted the linear dynamic feature over the dose range studied. The part of pharmacokinetic parameters of

Table 3
Main pharmacokinetic parameters of mildronate following single dose of 250, 500 and 1000 mg and multiple dose of 500 mg to Chinese healthy volunteers, respectively ($n = 10$, mean ± S.D.).

Parameters	250 mg	500 mg	1000 mg	500 mg (ss)
C_{max} (μg/mL)	11.00 ± 2.29	22.71 ± 3.95	39.30 ± 6.06	25.50 ± 3.63
T_{max} (h)	1.00 ± 0.00	0.88 ± 0.13	0.85 ± 0.13	0.95 ± 0.11
$t_{1/2}$ (h)	5.56 ± 1.55	6.46 ± 1.07	6.55 ± 1.17	15.34 ± 3.14
MRT (h)	3.51 ± 0.57	4.73 ± 0.85	4.66 ± 0.73	15.47 ± 3.06
AUC_{0-24} (mg h/L)	21.89 ± 4.76	46.32 ± 9.91	82.27 ± 17.07	58.56 ± 5.57
$AUC_{0-\infty}$ (mg h/L)	22.25 ± 4.90	47.85 ± 10.06	85.40 ± 17.99	76.26 ± 9.55
CL/F (L/h)	11.80 ± 2.88	10.86 ± 2.22	12.17 ± 2.46	6.65 ± 0.87
Vd/F (L)	93.76 ± 28.95	101.34 ± 26.32	114.55 ± 30.18	145.10 ± 23.96

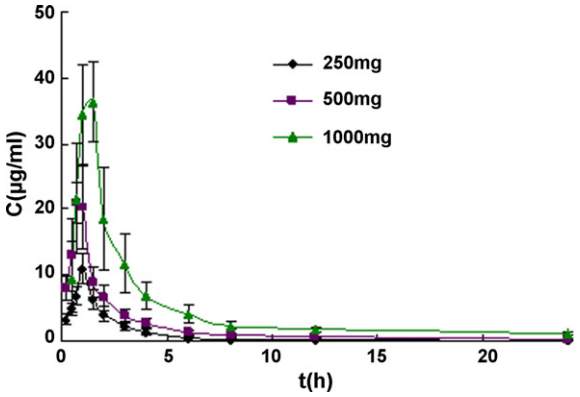

Fig. 4. Mean plasma concentration–time profile of mildronate in Chinese subjects following single dose of mildronate injection 250,500 and 1000 mg ($n = 10$). Each point represents mean + S.D.

multidoses are listed in Table 3, and the other parameters were as follows: C_{ssmin} (1.30 ±0.13) μg/mL; C_{av} (3.78 ± 0.35) μg/mL; DF (6.38 ± 0.54); AUC_{ss} (45.41 ± 4.17) mg h/L. The $t_{1/2}$ and AUC of multidoses were different from those of single-dose significantly ($P < 0.01$). The $t_{1/2}$ of single dose and multidoses were (6.46 ± 1.07) and (15.34 ± 3.14) h, respectively; The AUC_{0-24} of single dose and multidoses were (46.32 ± 9.91) and (58.56 ± 5.57) mg h/L, respectively. These findings suggested that accumulation of mildronate in plasma occurred.

4. Conclusion

A simple, rapid and sensitive method was developed for the determination of mildronate in human plasma and used to compare the pharmacokinetic parameters after single dose and multidoses intravenous infusion. The pharmacokinetic characteristics of mildronate after single dose in the healthy volunteers were based on first-order kinetics over the dose range tested. Differences in the pharmacokinetic parameters ($t_{1/2}$ and AUC) of mildronate between multidoses and single dose were significant, indicating the occurrence of accumulation in multidoses. The results suggest that decrease of the dosage or prolongation of the dosing interval should be considered in the phase II clinical trials. There were no adverse events at any dose during the conduct of the study and the drug was well tolerated without significant side effects.

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