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Journal of Chromatography B, 852 (2007) 35–39
JOURNAL OF CHROMATOGRAPHY B
www.elsevier.com/locate/chromb

Determination of mildronate in human plasma and urine by liquid chromatography–tandem mass spectrometry

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Received 21 November 2006; accepted 28 December 2006
Available online 3 January 2007

Abstract

A sensitive and selective analytical method based on liquid chromatography–triple–quadrupole mass spectrometer has been developed to determine mildronate in human plasma and urine. The aim of this work was to find a valid method to study the pharmacokinetic profiles of mildronate in humans. Mildronate is a heart protection medicine, a carnitine's structural analogue, so levocarnitine was used as an internal standard for quantification. Under the electrospray ionization source positive ion mode, calibration curves with good linearities ($r = 0.9998$ for plasma sample and $r = 0.9999$ for urine sample) were obtained in the range of 1.0–20,000 ng ml^{-1} for mildronate. The detection limit was 1 ng ml^{-1}. Recoveries were around 90% for the extraction from human plasma, and good precision and accuracy were achieved. This method is feasible for the evaluation of pharmacokinetic profiles of mildronate in humans, and to the best of our knowledge, this is the first report on LC–MS–MS analysis of mildronate in plasma and urine.

Keywords: Mildronate; LC–MS–MS

1. Introduction

Mildronate (also known as THP, MET-88) is a new type of heart protection medicine, a carnitine's structural analogue [1], developed by Latvia Organic Synthetic Inst., which was protectively sold in 1989 by the Grindeks Company in former Soviet Union and put into market in the Russian Federation in 2002 with the dosage in the form of capsule (250 mg, 500 mg) and injectable preparation (500 mg ml^{-1}). It lowers the intracellular concentration of free carnitine and thus suppresses fatty acid oxidation and facilitates glycolysis during ischemia [2,3].

To perform the pharmacokinetic study of mildronate in humans, a sensitive, accurate and stable method to determine the drug concentrations in plasma and urine is critical and essential. 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], while evaporative light scattering detection (ELSD) is inadequate for testing biological samples because the proteins and other interferent in biological samples will interfere the detection. At the same time, because of the strong polarity, mildronate is hard to separate from the endogenous substances in plasma by HPLC system without derivatization or radioisotopic exchange [5,6]. Thus, HPLC–MS–MS (HPLC coupled with triple–quadrupole mass spectrometer) analytical system enables the determination of mildronate with good selectivity and accuracy by its selected reaction monitoring (SRM).

This paper describes a sensitive and highly selective HPLC–MS–MS method used to determine mildronate in human plasma and urine that has never been reported.

2. Experimental

2.1. Solvents and reagents

Methanol (MeOH) was of HPLC-grade (J.T. Baker, USA). Water was prepared on a Milli-Q Water Purification System (Millipore, USA). Human plasma was obtained from Beijing Red Cross Blood Center (China).

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doi:10.1016/j.jchromb.2006.12.031

2.2. Sample preparation

2.2.1. Preparation of standard solutions

Pure reference mildronate (50 mg, accurately weighted) was dissolved in water in a 50-ml volumetric flask. This solution was diluted by human plasma and urine to give standard solutions for the calibration curves in the range of 1–20,000 ng ml^{-1} on-column ($n = 11$) for mildronate. 10 ng ml^{-1}, 500 ng ml^{-1}, 5000 ng ml^{-1} and 15000 ng ml^{-1} solutions were taken as the quality control (QC).

Pure reference levocarnitine (10 mg, accurately weighted) was dissolved in water in a 100-ml volumetric flask as the internal standard (IS) solution.

2.2.2. Preparation of the plasma sample

Precipitation method was used in the preparation of the plasma sample. A 50 μl internal standard solution and 2 ml methanol were added to 300 μl plasma sample; the mixture oscillated for 3 min and centrifuged at 3000 rpm for 10 min (Biofuge Pico-high Speed Centrifuge, USA); the supernatant was evaporated to dryness under nitrogen under 37 °C (Piece Reacti-ThermIIITM, USA); the dried sample was reconstituted in 300 μl mobile phase, oscillated for 3 min and centrifuged at 12,000 rpm for 10 min; the supernatant was separated and 10 μl of it was injected onto the HPLC column.

2.2.3. Preparation of the urine sample

A 100 μl urine sample was fortified with 10 μl of internal standard solution. Samples were briefly vortex-mixed and centrifuged to remove large particles. A 10 μl of supernatant was injected onto the HPLC column.

2.3. Instrument

All experiments were carried out on an API 3000 tandem quadrupole mass spectrometer (MS/MS) (Applied Biosystem, USA) equipped with an electrospray ionization source and interfaced to a hp1100 high-performance liquid chromatograph (Angilent, USA). All data were acquired and analysed using Analyst data processing software.

Chromatographic conditions were as follows: column, Inersil NH3, 5 μm, 250 mm × 4.6 mm (GL Sciences Inc., Tokyo, Japan); the mobile phase, water (60%) and methanol (40%); the flow-rate, 500 μl/min; temperature, 25 °C.

MS/MS conditions were as follows: the compounds were ionized using electrospray ion source in the positive mode, ion spray voltage was 4.0 kV, the capillary temperature was 300 °C and the pressure of the nebulizing gas (N_2) was 80 psi. The tandem mass spectrometer operated in selected reaction monitoring (SRM) mode. The collision gas (N_2) pressure was 40 psi, DP voltage was 20.0 V and collision-induced dissociation (CID) voltage was 40.0 V for mildronate and 23.0 V for levocarnitine. Selected reaction monitoring producing the ion combinations of *m/z* 147, 58 and *m/z* 162, 103 were used to quantify mildronate and the internal standard, respectively.

2.4. Method validation

Plasma and urine samples were quantified using the ratio of the peak area of mildronate to that of levocarnitine as the assay response. To evaluate linearity, plasma and urine calibration curves were prepared and assayed in triplicate on 3 consecutive validation days. Accuracy and precision were also assessed by determining QC samples at four concentration levels on the 5 different validation days. The accuracy was expressed by RE and the precision by R.S.D. The extraction recoveries of mildronate at four QC levels were determined.

3. Results and discussion

3.1. Selectivity

Under the present chromatographic conditions described in Section 2, no endogenous interfering or late eluting peaks were found. Typical retention time for mildronate was 7.26 min and for the internal standard levocarnitine was 7.50 min. Fig. 1 displays the product ion spectra of $[M+H]^+$ ions from mildronate and $[M]^+$ ions from levocarnitine, respectively. Mildronate showed an intense ion at *m/z* 147 and levocarnitine at *m/z* 162, which was chosen, in the selected reaction monitoring (SRM) acquisition for mildronate and levocarnitine, respectively.

The most suitable collision energy was determined by observing the maximum response obtained for the fragment ion peak *m/z*. The product ion of mildronate used for SRM acquisition was *m/z* 58 ions and of levocarnitine was *m/z* 103 ions. Fig. 2 shows representative chromatograms of mildronate and levocarnitine.

3.2. Linearity and lower limit of quantification

The peak area ratios of mildronate to that of levocarnitine were used for the construction of calibration curves, using $1/\chi^2$ weighted linear least-squares regression of plasma and urine concentrations over the range of 1.0–20,000 ng ml^{-1} examined in plasma and urine.Typical regression equation and correlation coefficient of plasma sample are $\gamma = 0.0007X + 0.1067$ ($r = 0.9998$), and that of urine sample are $\gamma = 0.0008X + 0.0544$ ($r = 0.9999$), where γ is the peak area ratio of mildronate to levocarnitine, and X is the concentration of mildronate in sample. The results of the linearity are shown in Tables 1 and 2. The lower limit of quantification of mildronate was 1 ng ml^{-1} with an accuracy within 5% in terms of relative error (RE), and a precision in terms of relative standard deviation (R.S.D.) was ≤ 5% [7].

3.3. Extraction recovery and stability

Recovery of plasma samples was assessed by determining QC samples at 10 ng ml^{-1}, 500 ng ml^{-1}, 5000 ng ml^{-1} and 15000 ng ml^{-1}, each five times and the peak area of the extracted QC samples to that of the same concentration pure water solution of mildronate were compared without extraction. The mean extraction recoveries of mildronate determined at that four concentrations in human plasma are shown in Table 3.

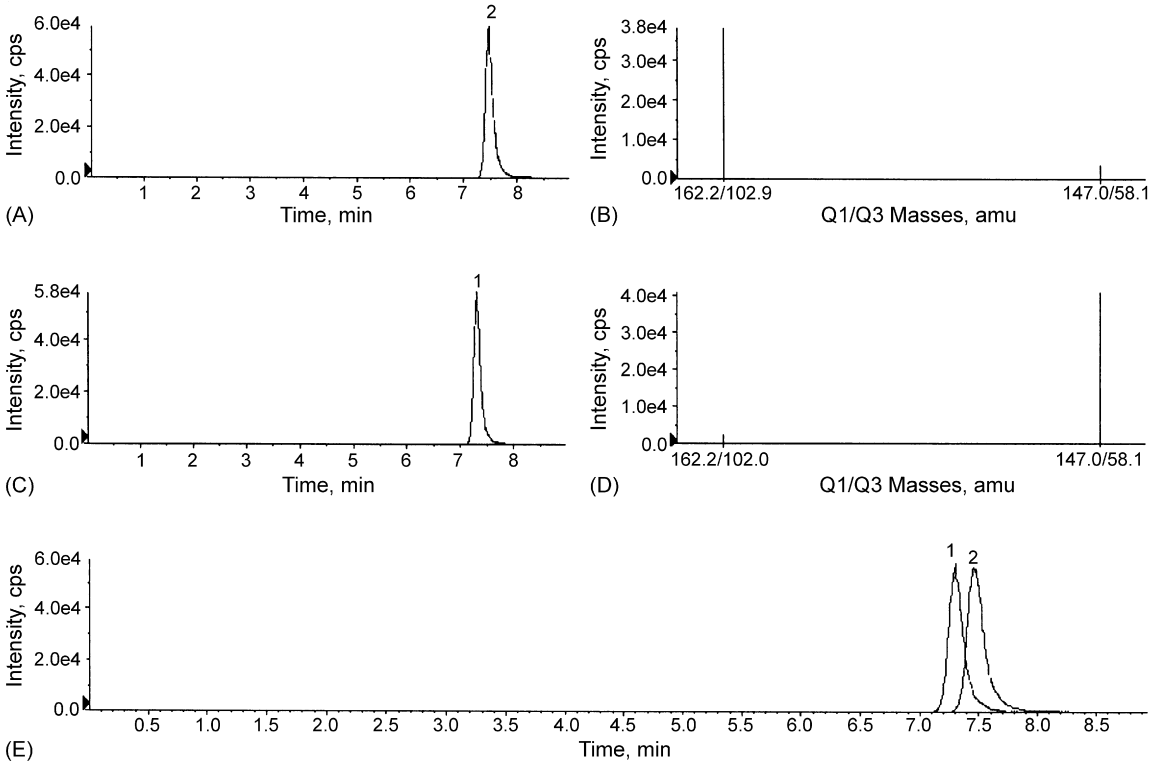

Fig. 1. Chromatogram and mass spectra of mildronate and IS. (A) Chromatogram of IS; (B) production mass spectra of $[M]^+$ of IS; (C) chromatogram of mildronate; (D) production mass spectra of $[M+H]^+$ of mildronate; (E) chromatogram of mildronate and IS (1: mildronate; 2: IS).

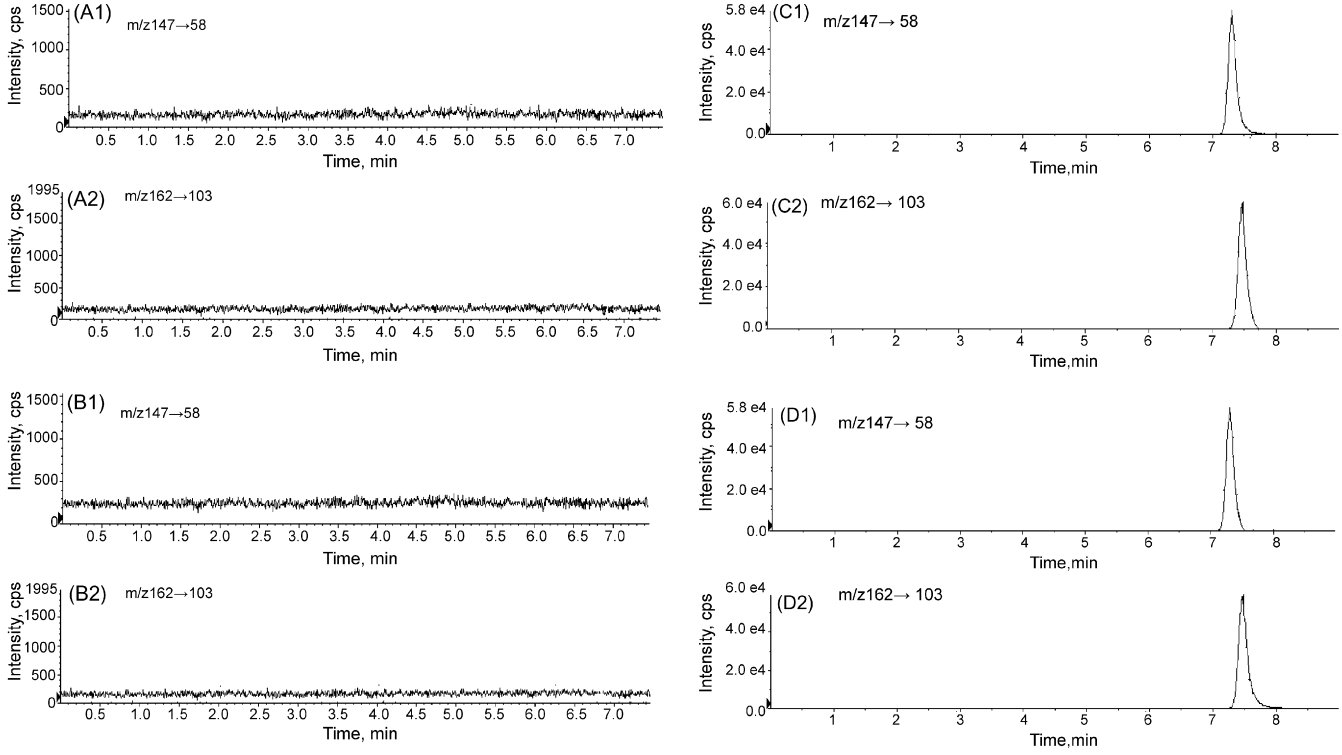

Fig. 2. Representative selected reaction monitoring chromatograms of mildronate and IS in plasma and urine. (A1) Blank plasma under *m/z* 147 and 58 monitoring; (A2) black plasma under *m/z* 162 and 103 monitoring; (B1) blank urine under *m/z* 147 and 58 monitoring; (B2) blank urine under *m/z* 162 and 103 monitoring; (C1) mildronate in plasma; (C2) IS in plasma; (D1) mildronate in urine; (D2) IS in urine.

Table 1
Calibration curve data for mildronate in plasma

Calibration standard concentration (ng ml^{-1})	Calculated concentration (mean ± S.D., n = 6) (ng ml^{-1})	R.S.D. (%)	Deviation (%)
1	0.95 ± 0.041	4.3	−4.66
5	5.20 ± 0.182	3.5	4.07
10	9.50 ± 0.238	2.5	−4.96
50	47.58 ± 1.760	3.7	−4.84
100	95.33 ± 1.811	1.9	−4.67
500	479.00 ± 15.81	3.3	−4.20
1,000	975.87 ± 40.01	4.1	−2.41
2,000	1957.81 ± 41.11	2.1	−2.11
5,000	5054.39 ± 60.65	1.2	1.09
10,000	10263.67 ± 328.4	3.2	2.64
20,000	20044.07 ± 300.6	1.5	0.22

Table 2
Calibration curve data for mildronate in urine

Calibration standard concentration (ng ml^{-1})	Calculated concentration (mean ± S.D., n = 6) (ng ml^{-1})	R.S.D. (%)	Deviation (%)
1	0.96 ± 0.031	3.2	−4.49
5	5.19 ± 0.161	3.1	3.71
10	9.78 ± 0.411	4.2	−2.25
50	48.23 ± 1.495	3.1	−3.54
100	95.88 ± 1.342	1.4	−4.12
500	491.49 ± 17.69	3.6	−1.70
1,000	957.30 ± 41.16	4.3	−4.27
2,000	1916.32 ± 24.91	1.3	−4.18
5,000	4787.46 ± 76.60	1.6	−4.25
10,000	9805.68 ± 382.4	3.9	−1.94
20,000	19317.40 ± 444.3	2.3	−3.41

During the experiment, we found that mildronate was stable for at least 24 h at room temperature under experimental conditions. QC samples at 10 ng ml^{-1}, 500 ng ml^{-1}, 5000 ng ml^{-1} and 15000 ng ml^{-1} were kept at room temperature and detected at 0 h, 4 h, 8 h, 12 h and 24 h, the R.S.D.% of the five times of the detection results of the four QC samples from low concentration to high are 0.85%, 0.95%, 0.68%, 0.65% for plasma samples and 0.97%, 0.78%, 0.70%, 0.89% for urine samples.

Also, mildronate was stable in plasma and urine after five freeze-and-thaw cycles. The QC samples were put into −70 °C refrigerator for 2 h and taken out to melt in room temperature; this freeze-and-thaw operation was repeated five times, and the samples were detected before the first freeze-and-thaw operation and each time after they were melted. The R.S.D.% of the six times of the detection results of the four QC samples from low concentration to high are 0.67%, 0.93%, 0.98%, 0.76% for plasma samples and 0.77%, 0.74%, 0.83%, 0.90% for urine samples.

Table 3
Recoveries of extraction method of mildronate in human plasma (n = 5)

Concentration (ng ml^{-1})	Recovery (%)	R.S.D.%
10	89.4	2.5
500	90.2	2.3
5,000	92.0	1.9
15,000	92.6	1.4

3.4. Precision and accuracy

Precision and accuracy were assessed by determining QC samples at 10 ng ml^{-1}, 500 ng ml^{-1}, 5000 ng ml^{-1} and 15000 ng ml^{-1} on 5 different validation days. The intra-run and inter-run precision ranged from 0.84% to 1.2% and from 1.0% to 1.6% for each QC level, respectively. The accuracy was within 1.8%. The data calculated using one-way ANOVA indicated that the values were within the acceptable range and the method was accurate and precise [8].

4. Conclusion and discussion

The present method for the determination of mildronate in human plasma and urine has proved to be rapid, sensitive, and selective, and it requires relatively small volumes of sample; it is suitable for the pharmacokinetic study of mildronate in humans. To date, no application of LC–MS–MS for analysis of mildronate in human plasma and urine has been reported.

Sample pretreatment procedures for plasma and urine were characterized by easy-to-use methods and speed. They provided a sufficient clean up of the biological samples prior to LC–MS–MS analysis and showed no significant loss of the analytes during sample handling.

By selected reaction monitoring of MS–MS, a precursor ion was isolated and fragmented by CID. Subsequently, one or more product ions were isolated and scanned, allowing specific precursor–product ion transitions to follow and sensitivity

by total elimination of background noise to increase, and the high sensitivity permitted measurement of low concentration of analytes of interest, eliminating the need for pre-concentration during sample pretreatment.

Structural analogue levocarnitine proved to be an excellent internal standard for calibration, compensating for variations in sample handling, instrument parameters and matrix effect.

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