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Determination of L-threonate in human plasma and urine by high performance liquid chromatography-tandem mass spectrometry

Hongyun Wang, Ji Jiang, Pei Hu *

Clinical Pharmacology Research Center, Peking Union Medical College Hospital and Chinese Academy of Medical Sciences, Beijing 100730, China

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Abstract

A fast and selective HPLC-MS–MS method was established to determine L-threonate in human plasma and urine. Plasma and urine samples were extracted by protein precipitation and diluted with water, then chromatographed on an YMC J'Sphere C_{18} column with methanol–acetonitrile–10 mM ammonium acetate (20:5:75, v/v) as mobile phase, and at a flow rate of 0.2 ml/min. Detection was performed on a triple–quadrupole tandem mass spectrometer using negative electrospray ionization (ESI). Multiple reactions monitoring (MRM) was used and L-threonate was quantified by monitoring the ion transition of m/z 134.5 → 74.7. The linear calibration curves of L-threonate in plasma and urine were obtained over the concentration range of 0.25–50 μg/ml and 2.5–500 μg/ml, respectively. Lower limit of quantitation was 0.25 and 2.5 μg/ml, respectively. Accuracy was within 85–115%, and intra- and inter-batch precision (R.S.D.%) were within ±15%. The method proved to be accurate and specific, and was applied to the pharmacokinetic study of L-threonate in Chinese healthy subjects.

Keywords: L-Threonate; HPLC-MS–MS; MRM

1. Introduction

Calcium L-threonate is a novel drug with L-threonic acid as its calcium carrier, and it is developed for the treatment of osteoporosis and calcium supplement. L-Threonate is an active degradation product of Vitamin C, and it can improve the proliferation of osteoblasts and enhance production of mineralized nodules and collagenous protein [1–3]. The whole pharmacological activity of calcium L-threonate on osteoporosis deemed to be the cooperation of L-threonate and calcium.

Few references are available about the determination of L-threonate except a capillary electrophoresis method and a GC–MS method [4,5]. The aim of this paper is to establish a reliable HPLC-MS–MS method for the quantitation of L-threonate in human plasma and urine. It needs to be pointed out that L-threonate is an endogenous substance as a degradation product of Vitamin C, and the latter is inevitably ingested by people in their daily life. During the course of method development, in order to eliminate the endogenous interference, human albumin and saline solution (0.9% NaCl) were used to replace blank plasma and urine. The method proved to be effective, sensitive and specific, and can be applied to the pharmacokinetic study of L-threonate in Chinese healthy subjects.

2. Experimental

2.1. Chemicals and reagents

Calcium L-threonate was provided by Asian Ju Neng pharmaceutical Co. Ltd. (China) (Fig. 1). Methanol and acetonitrile (HPLC grade) were purchased from Fisher (Fair Lawn, NJ, USA). Ammonium acetate (analytical grade) was purchased from Peking Chemical Plant (Beijing, China). Human albumin (20%) was produced by HuaLan life science institute (Beijing, China). 0.9% NaCl was supplied by Peking Union Medical College Hospital. Blank human plasma and urine were provided by the enrolled healthy subjects. Distilled water was prepared with a Milli-Q water purifying system (Millipore, Bedford, USA).

* Corresponding author. Tel.: +86 10 6529 6576; fax: +86 10 6529 6573.
E-mail address: peihu@public3.bta.net.cn (P. Hu).

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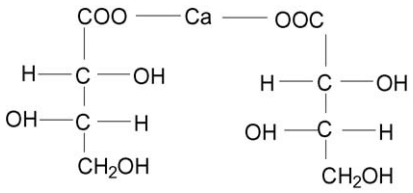

Fig. 1. Chemical structure of calcium L-threonate.

2.2. HPLC conditions

HPLC system consisted of Waters 2790 pump and autosampler (Waters, USA). The analytical column was YMC J'Sphere C_{18} column (50 mm × 4.6 mm i.d., 3.5 μm) coupled with a C_{18} (YMC) guard column (10 mm × 4.6 mm i.d., 3.5 μm), and the column temperature was set at 30 °C. The mobile phase was composed of methanol–acetonitrile–10 mM ammonium acetate (20:5:75, v/v). The flow rate was 0.2 ml/min and the injection volume was 20 μl.

2.3. Mass spectrometer conditions

Quattro Ultima triple–quadruple mass spectrometer (Micromass) equipped with ESI was used, and the detection was performed in negative ionization mode. Capillary voltage and cone voltage were set at −2300 and −25 V, respectively. The temperature of ion source was 120 °C with ultrahigh-purity nitrogen as cone gas (120 l/h) and nebulizer gas (adjusted to the upper limit). Desolvation gas was heated to 350 °C and set at a flow rate of 650 l/h. With argon as collision gas (2.6 mTorr), multiple reaction monitoring (MRM) was applied to detect L-threonate by monitoring the ion transition of *m/z* 134.5 → 74.7, and collision energy was set at 15V. Product ion spectrum of L-threonate was shown in Fig. 2.

2.4. Preparation of calibration curve and QC samples in plasma and urine substitutes

L-Threonate is an endogenous substance as a degradation product of Vitamin C. Human albumin (4.5% ALB) and saline solution (0.9% NaCl) were respectively used as blank plasma and urine substitute in this study. Blank plasma substitute was made by diluting concentrated human serum albumin (20%) with saline. *Note*: Normal range of human albumin in Chinese was 3.8–5.1%, average value (4.5%) was used here [6].

Stock solution for L-threonate was prepared by dissolving accurately weighted standard compound (calcium L-threonate) with distilled water to give the final concentration of 1 mg/ml, and stored at −30 °C. Calibration curves and QC samples in plasma and urine were prepared by diluting the stock solutions (1 mg/ml) with plasma substitute and urine substitute. The final concentrations of calibration curve samples were 0.25, 0.5, 1, 2.5, 5, 10, 25 and 50 μg/ml in plasma substitute, and 2.5, 5, 10, 25, 50, 10, 250 and 500 μg/ml in urine substitute, respectively. The final concentrations of QC samples were 0.75, 12.5 and 40 μg/ml in plasma substitute, and 7.5, 125 and 400 μg/ml in urine substitute, respectively. All the biological samples were stored at −30 °C until analysis.

2.5. Extraction procedure of plasma and urine samples

To each 0.1 ml volume of plasma sample, 0.3 ml of methanol was added and vortexed for 1 min, then the tubes containing samples were capped and centrifuged at 13,000 rpm for 5 min. After plasma protein was precipitated, 0.2 ml of clean supernatant was collected and diluted with 1.5 ml of distilled water, and then 20 μl of the diluted sample was injected.

To each 0.1 ml volume of urine sample, 2 ml of distilled water was directly added and vortexed for 1 min, then 20 μl of the diluted sample was injected.

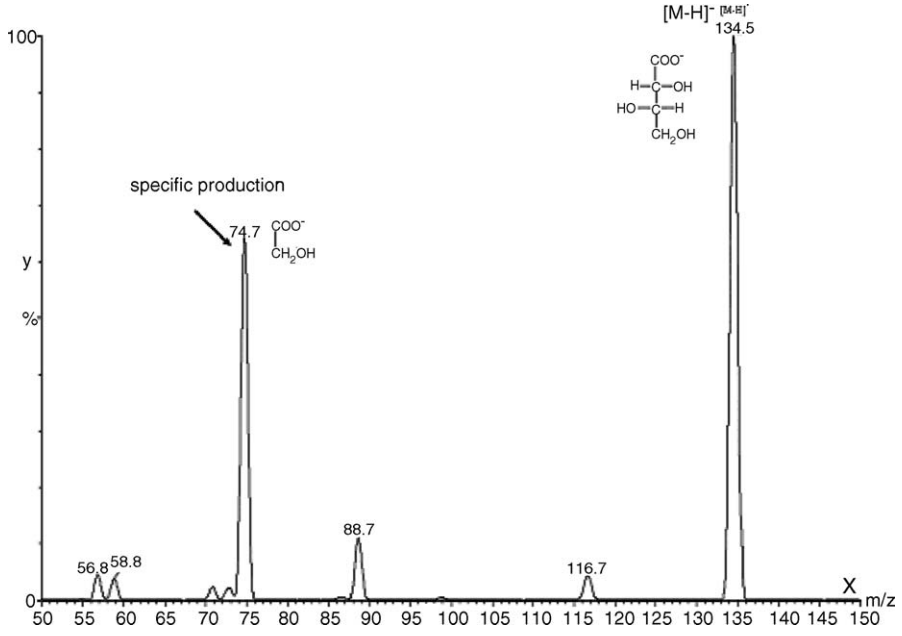

Fig. 2. Product ions spectrum of L-threonate.

2.6. Method validation

Calibration curve samples were prepared in duplicate every batch for five independent batches. Linear equations (weighted 1/concentration) between the concentrations and the response (peak area) were constructed. The lower limit of quantitation (LLOQ) of L-threonate in plasma and urine substitute was set at the concentration of the lowest non-zero calibration samples. Intra- and inter-batch precision and accuracy were determined by assessing QC samples at low, medium and high concentration in five independent analysis batches. The extraction recovery of L-threonate in plasma substitute was determined by calculating the peak area of extracted low, medium and high QC samples against unextracted standards solutions at the same concentration representing 100% recovery. The short-term stability of L-threonate in plasma substitute was determined by assessing replicate QC samples, which were kept at room temperature (25 °C) for 24 h. Freeze–thaw stability was studied after three cycles, and long-term stability was done by assessing QC samples stored at −30 °C for 3 months. In assessing the processed sample stability of L-threonate in plasma substitute, QC samples were extracted and injected, then subsequently re-injected after storage at 25 °C for 24 h. The stock solution stability of L-threonate was determined by comparing freshly prepared stock solution to the solution, which was stored at −30 °C for 6 months.

2.7. Influence of substitute matrix on extraction recovery

Calibration curves samples were prepared by spiking the stock solution (1 mg/ml) with blank plasma and 4.5% ALB, urine and saline ($n = 6$). The final concentrations in plasma and 4.5% ALB were 0.25, 0.5, 1, 2.5, 5, 10, 25 and 50 μg/ml, and 2.5, 5, 10, 25, 50, 10, 250 and 500 μg/ml in urine and saline, respectively. After these samples were analyzed, the linear slopes of peak area versus concentration in different matrixes were compared using independent-samples test.

2.8. Data acquisition and analysis

Data acquisition was performed using MassLynx 3.5 software (Micromass). Peak integration and calibration were processed with QuanLynx 3.5 software. A linear equation (weighted by 1/concentration) was utilized to determine the relationship between concentration and peak area of L-threonate (external standard quantitation). Concentrations of QC and unknown samples were calculated by interpolation from the equations. SPSS

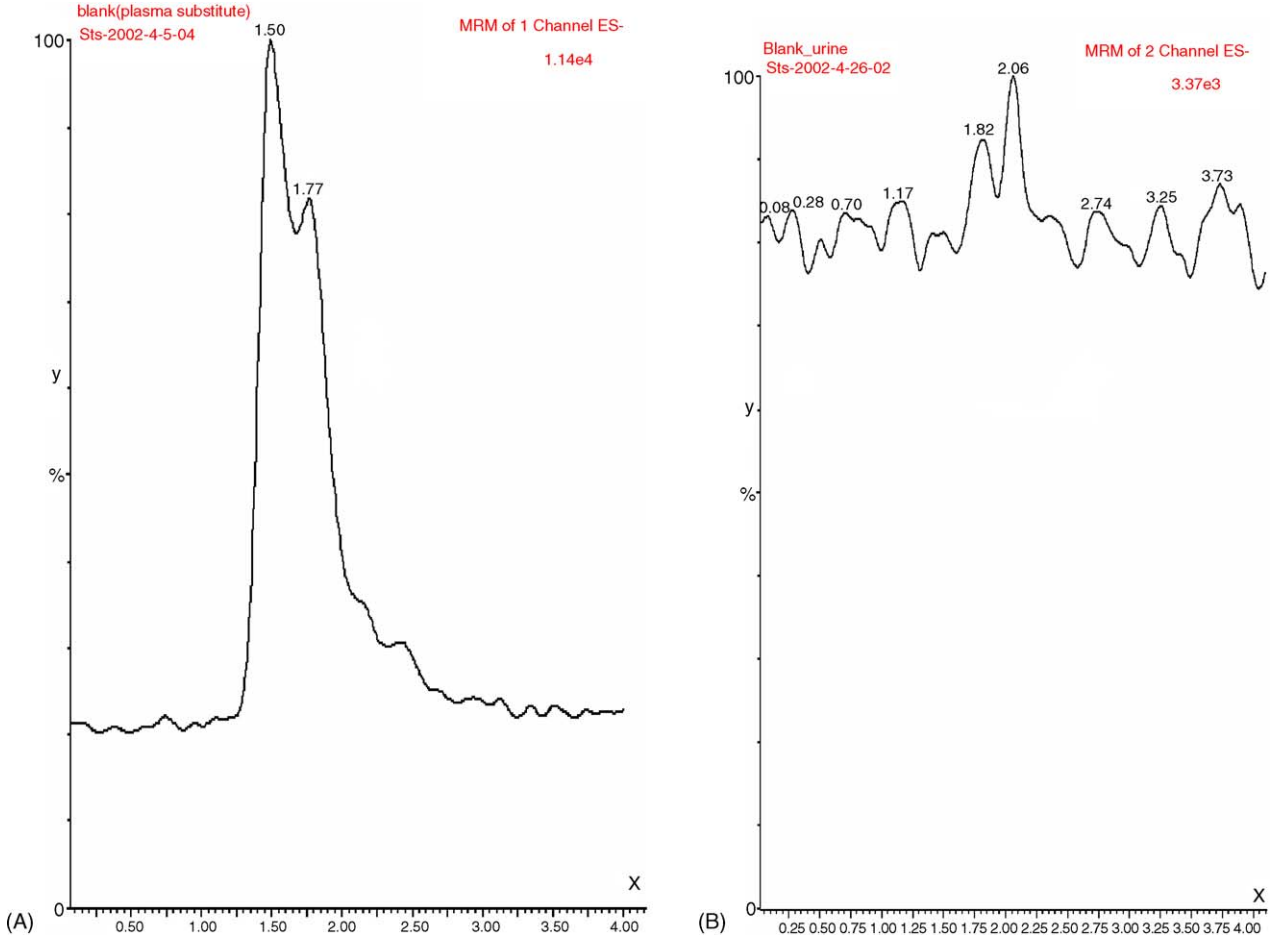

Fig. 3. Representative MRM chromatograms of extracted blank plasma sample without drug added: (A) blank plasma substitute and (B) blank urine substitute.

11.0 software was used to process statistical analysis, and statistical significance was concluded if $P < 0.05$.

3. Results and discussion

3.1. HPLC-MS–MS method development

HPLC-MS–MS is a powerful technique, and now is widely used in biological analysis. During the method development of L-threonate, standard solution (2 μg/ml) was introduced into mass spectrometer using syringe pump at a flow rate of 5 μl/min. Because L-threonate is an acidic compound, negative ionization mode was preferred. ESI parameters such as capillary voltage, cone voltage and nebulizer gas were optimized to obtain strong signal of $[M-H]^-$ at *m/z* 134.5. High specific MRM scan was used, and the most strong fragment ion at *m/z* 74.7 was selected as the product ion used in MRM acquisition. Collision energy and argon gas flow were determined when observing maximum response of the product ion. A product ion spectrum of L-threonate was shown in Fig. 2.

Because L-threonate is easily soluble in water, extraction method such as solid phase extraction (SPE) and protein precipitation are preferable than liquid–liquid extraction. During the method development, protein precipitation was firstly tested, and accurate determination could be done when the extracts were injected into HPLC-MS–MS system. Finally, plasma samples were extracted by protein precipitation, and urine samples were directly diluted with water. Internal standard was not necessary in this study. The method proved to be timesaving and robust, compatible with the fast analysis possible with HPLC-MS–MS, and therefore offering high sample throughput.

3.2. Method validation

3.2.1. Specificity

Representative MRM chromatograms of extracted blank samples were shown in Fig. 3. Representative chromatograms of extracted QC samples and unknown samples from a subject after oral administration of 675 mg calcium L-threonate were shown in Figs. 4 and 5, respectively. It was demonstrated that there was no significant interference in blank urine substitute, but an interfering peak eluted at the retention time of L-threonate in blank plasma substitute. However, Peak area of the interfering substance was only 8.1% of LLOQ (Table 1), so the tiny interference could be ignored. It was concluded that quantitative determination of L-threonate could not be interfered.

3.2.2. Calibration curve

Calibration curves of L-threonate in plasma substitute and urine substitute were validated over the concentration range of 0.25–50 μg/ml and 2.5–500 μg/ml, respectively. Typical equations of calibration curves were as followed ($n = 10$):

Plasma substitute :

$$Y = 1.3873 \times 10^4 X + 4.6501 \times 10^3, \qquad r = 0.9990$$

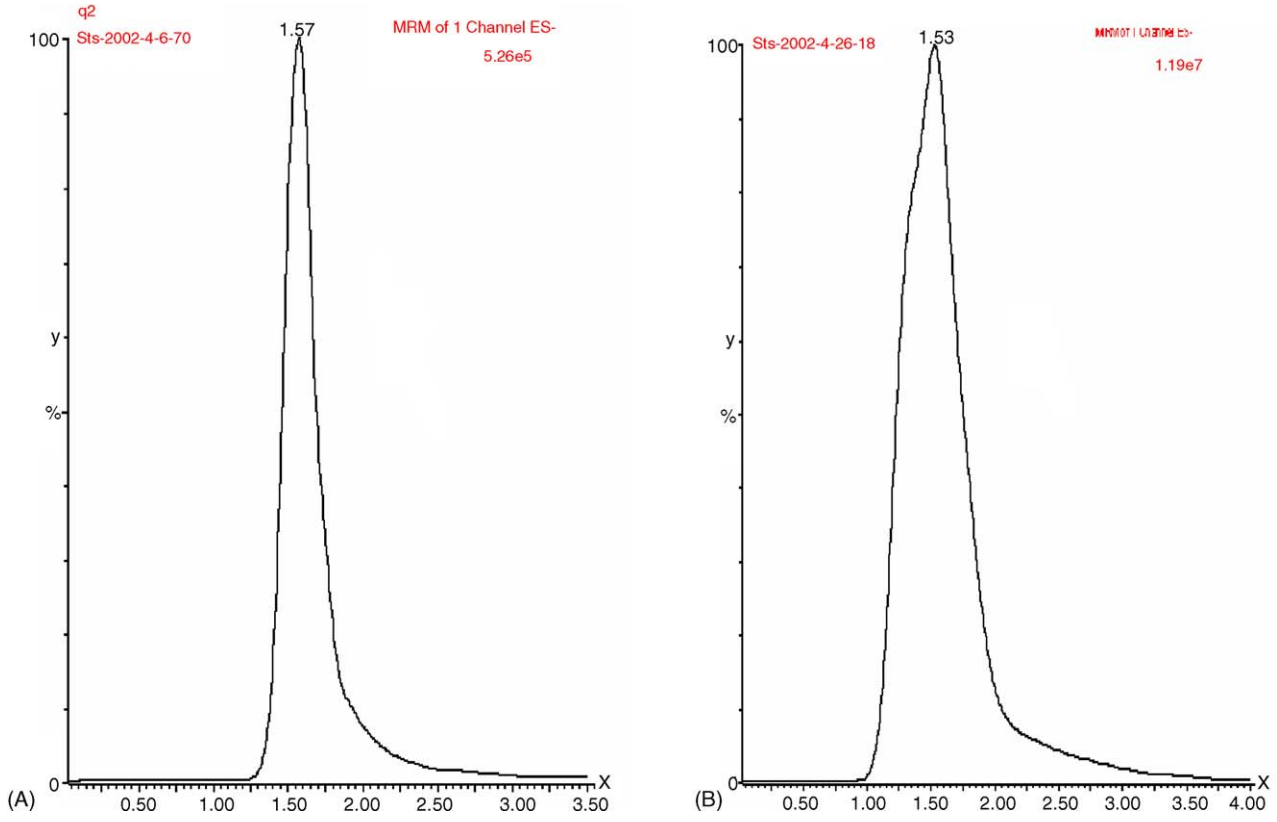

Fig. 4. Representative MRM chromatograms of QC sample at medium concentration: (A) plasma substitute (12.5 μg/ml) and (B) urine substitute (125 μg/ml).

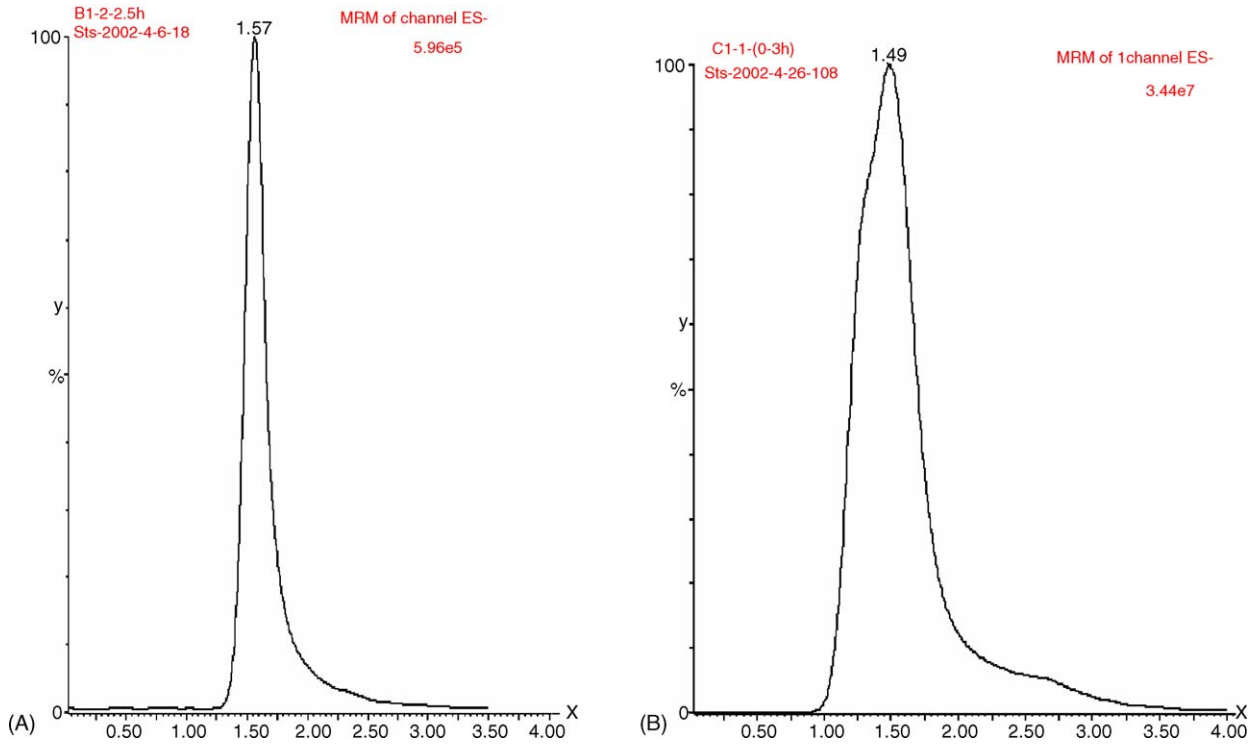

Fig. 5. Representative MRM chromatograms of unknown sample from a subject after oral administration of 675 mg calcium L-threonate: (A) plasma sample drawn at 2.5 h after administration (10.3 μg/ml) and (B) urine samples collected between 0 and 3 h after administration (110.1 μg/ml).

Urine substitute :

$$Y = 5.7804 \times 104X + 2.5339 \times 10^5, \qquad r = 0.9968$$

Here Y represented peak area, and X represented drug concentration. Back-calculated concentrations of calibration curve samples were within 85–115% of their nominal values.

3.2.3. Precision and accuracy

Low, medium and high QC samples in plasma and urine substitute were assayed in five independent batches, and the results were used to evaluate precision (R.S.D.%) and accuracy (%nominal). Accuracy was calculated according to the equation: accuracy (%) = [(measured concentration/nominal concentration) × 100]. Inter- and intra-batch precision of QC samples was all less than 15% and the accuracy was within 85–115% (Table 2).

Table 1
Comparison of blank plasma substitute and LLOQ samples ($n = 10$)

	Peak area of L-threontae	
	Blank plasma substitute	LLOQ
	3820.0	38300.0
	2980.0	42060.0
	4650.0	36100.0
	2230.0	44500.0
	3126.0	48700.0
	2689.0	36200.0
	4106.0	41300.0
	2988.0	36800.0
	3166.0	37260.0
	3260.0	48300.0
Mean	3301.5	40952.0
%LLOQ	8.1	NA[a]
S.D.	707.9	4853.3
R.S.D.%	21.4	11.9

[a] Not available.

3.2.4. Extraction recovery

Extraction recovery of L-threonate in plasma substitute was determined by calculating the peak area of extracted low, medium and high QC samples against unextracted standards at the same concentration representing 100% recovery. The extraction recovery of low, medium and high QC samples was on average 55.6, 56.9 and 60.0%, respectively (Table 2).

The influence of substitute matrix on extraction recovery was tested by comparing linear slopes in different matrix (Table 3). The linear slopes in plasma were not statistically different from the slopes in 4.5% ALB ($P > 0.05$, $n = 6$), and also were not statistically different between urine and saline ($P > 0.05$, $n = 6$). Therefore, it was concluded that substitute matrixes had no influence on the extraction recovery.

3.2.5. Lower Limit of quantitation (LLOQ)

The lower limit of quantitation (LLOQ) of L-threonate was set at the concentration of the lowest non-zero calibration sample, 0.25 μg/ml for plasma and 2.5 μg/ml for urine, respectively. Six replicate LLOQ samples were assayed in five independent batches. Intra- and inter-batch precision (R.S.D.%) of LLOQ samples was on average 8.1 and 7.6% in plasma substitute, and

Table 2
Summary of precision, accuracy and extraction recovery of L-threonate in plasma and urine substitute

Nominal concentration (μg/ml)	Precision (R.S.D.%)			
	Accuracy (%), $n=30$	Inter-batch, $n=30$	Intra-batch, $n=5$	Extraction recovery (%), $n=6$
Plasma substitute				
0.75	100.0	6.1	9.4	55.6
12.5	98.0	8.5	11	56.9
40	103.9	5.9	8.8	60.0
Urine substitute				
7.5	107.3	3.8	5.2	ND[a]
125	108.3	6.3	6.9	ND
400	101.0	9.1	10.9	ND

[a] Not done.

Table 3
Linear slopes of the calibration curves spiked with different matrixes in the test for influence of substitute on extraction recovery ($n=6$)

	Plasma ($\times 10^4$)	ALB ($\times 10^4$)	Urine ($\times 10^4$)	Saline ($\times 10^4$)
	1.494	1.826	5.326	6.013
	1.812	1.789	5.889	5.113
	1.793	1.489	5.468	5.128
	1.662	1.592	5.991	5.326
	1.589	1.696	6.329	5.997
	1.941	1.756	6.012	5.882
Mean	1.715	1.691	5.836	5.577
S.D.	0.164	0.129	0.373	0.433
R.S.D..%	9.5	7.6	6.4	7.8

9.1 and 6.8% in urine substitute. The accuracy (%nominal) was on average 106.9 and 103.8% in plasma and urine substitutes, respectively. The present LLOQ was sensitive enough for the pharmacokinetic study of L-threonate although much lower limit could be reached. Representative chromatogram of LLOQ was showed in Fig. 6.

3.2.6. Stability

The stability of L-threonate was assessed under different storage and processing conditions. L-Threonate was found to be stable in plasma substitute for at least 24 h at room temperature, and was stable following three cycles of freeze–thaw. L-Threonate proved to be stable in plasma substitute for 3 months at −30 °C freezing condition, and was stable in extracts for 24 h at room temperature. Result of stock solution stability demonstrated that L-threonate was stable in distilled water for 6 months at −30 °C (Table 4).

Long-term of L-threonate in human plasma and urine were also performed. In this test, several plasma and urine samples from the subjects who completed the clinical trial were randomly selected and analyzed, the samples at the same blood-drawing time points were re-analyzed after they were stored at room

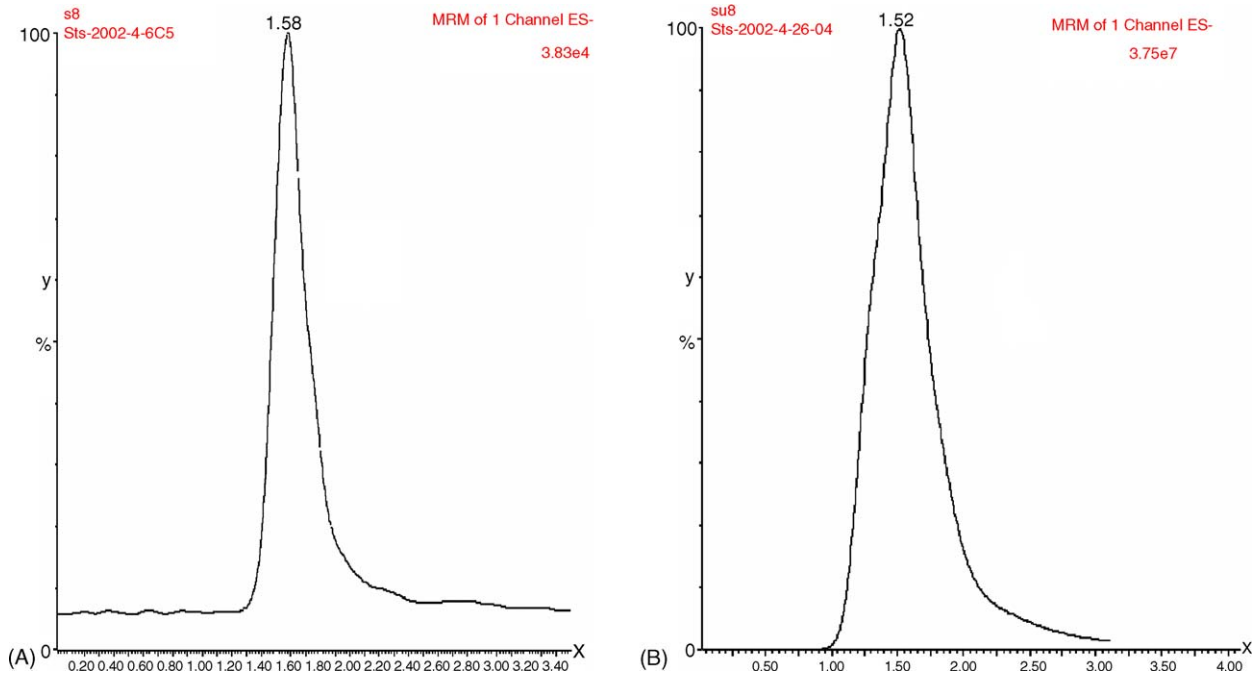

Fig. 6. Representative MRM chromatograms of LLOQ samples: (A) plasma substitute (0.25 μg/ml) and (B) urine substitute (2.5 μg/ml).

Table 4
Stability of L-threonate under different storage and process conditions ($n = 6$)

Nominal concentration (μg/ml)	Stability QC (mean ± S.D.) (μg/ml)	Freshly prepared QC (mean ± S.D) (μg/ml)	Difference (%)
Long-term stability			
0.75	0.66 ± 0.1	0.73 ± 0.0	−9.6
12.5	11.9 ± 0.9	12.8 ± 0.5	−7.0
40	42.2 ± 1.2	40.8 ± 2.1	3.4
Short-term stability			
0.75	0.70 ± 0.1	0.73 ± 0.0	−4.1
12.5	12.1 ± 1.2	12.8 ± 0.5	−5.4
40	41.8 ± 2.9	40.8 ± 2.1	2.5
Freeze–thaw stability			
0.75	0.78 ± 0.1	0.73 ± 0.0	6.9
12.5	13.2 ± 0.4	12.8 ± 0.5	3.1
40	42.7 ± 0.5	40.8 ± 2.1	4.7
Processed sample stability			
0.75	0.80 ± 0.1	0.73 ± 0.0	9.6
12.5	13.6 ± 1.1	12.8 ± 0.5	6.3
40	37.9 ± 4.5	40.8 ± 2.1	−7.1
Stock solution stability			
10[a]	65195.0 ± 21224.0[b]	71726.7 ± 18233.9[b]	−9.1

[a] The concentration of stock solution (1 mg/ml) was so high that direct injection could pollute the analytical instruments, so necessary dilution was done, and the stock solution was diluted to 10 μg/ml. Stock solution stability was finally determined by comparing the peak area of the diluted stock solution at 10 μg/ml against freshly prepared solution representing 100%.
[b] Peak area.

temperature for 24 h and at −30 °C for 3 months. The stability was assessed by comparing the two-time measured result. The results showed that L-threonate was stable in human plasma and urine during storage at room temperature for 24 h and at −30 °C for 3 months (Table 5).

3.3. Application in the pharmacokinetic study of L-threonate

This was a Phase I, open-label, randomized, cross-over design and dose-ranging study to assess the pharmacokinetics of L-threonate in healthy Chinese subjects. Twelve healthy subjects were enrolled and received a single oral dose of 675, 2025 and 4050 mg calcium L-threonate. Plasma and urine samples were collected and assayed with the validated HPLC-MS–MS method. Mean plasma concentration–time curves were shown in Fig. 7.

Table 5
Stability studies of L-threonate in human plasma and urine

	Batch 1	Batch 2	Difference (%)
Short-term stability			
Plasma samples			
Subject 2–0.5 h	6.8	7.6	11.8
Subject 5–1 h	13.6	12.6	−7.8
Subject 11–6 h	6.3	5.9	−5.5
Urine samples			
Subject 1–(0–3) h	110.1	98.6	−10.4
Subject 7–(12–24) h	11.1	12.1	8.7
Subject 9–(6–9) h	52.7	55.6	5.6
Long-term stability			
Plasma samples			
Subject 2–0.5 h	6.8	5.9	−13.2
Subject 5–1 h	13.6	12.3	−9.7
Subject 11–6 h	6.3	7.1	13.3
Urine samples			
Subject 1–(0–3) h	110.1	106.8	−3.0
Subject 7–(12–24) h	11.1	12	7.8
Subject 9–(6–9) h	52.7	56.1	6.5

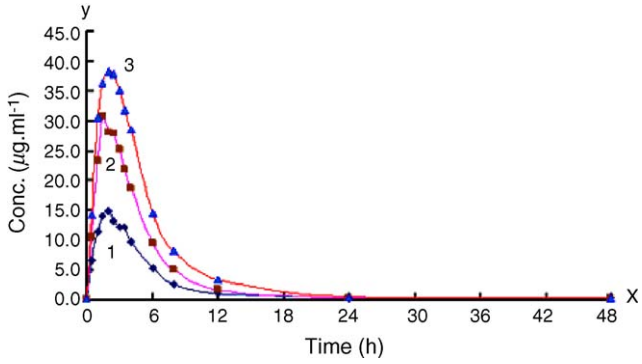

Fig. 7. Mean plasma concentration-time curves of L-threonate after single oral administration of 675 mg (1), 2025 mg (2) and 4050 mg (3) calcium L-threonate ($n = 12$).

4. Conclusion

A fast HPLC-MS–MS method has been developed and validated for the quantitative determination of L-threonate in human plasma and urine. The method proved to be timesaving, specific and accurate, and therefore offering high sample throughput. The method has been applied to the pharmacokinetic studies of L-threonate in Chinese healthy subjects.

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