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Development and validation of a liquid chromatography–tandem mass spectrometry method for the determination of xanthinol in human plasma and its application in a bioequivalence study of xanthinol nicotinate tablets

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ABSTRACT

A sensitive, rapid liquid chromatographic–electrospray ionization mass spectrometric method for the determination of xanthinol in human plasma was developed and validated. Xanthinol nicotinate in plasma (0.5 mL) was pretreated with 20% trichloroacetic acid for protein precipitation. The samples were separated using a Lichrospher silica (5 μm, 250 mm × 4.6 mm i.d.). A mobile phase of methanol–water containing 0.1% formic acid (50: 50, v/v) was used isocratically eluting at a flow rate of 1 mL/min. Xanthinol and its internal standard (IS), acyclovir, were measured by electrospray ion source in positive selected reaction monitoring mode. The method demonstrated that good linearity ranged from 10.27 to 1642.8 ng/mL with $r = 0.9956$. The limit of quantification for xanthinol in plasma was 10.27 ng/mL with good accuracy and precision. The mean plasma extraction recovery of xanthinol was in the range of 90.9–100.2%. The intra- and inter-batch variability values were less than 4.8% and 7.9% (relative standard deviation, R.S.D.), respectively. The established method has been successfully applied to a bioequivalence study of two xanthinol nicotinate tablets for 20 healthy volunteers.

1. Introduction

Xanthinol nicotinate is a compound of nicotinic acid and xanthinol (derivative of xanthine), which has been shown to increase brain glucose metabolism, improve brain ATP levels [1] and improve brain blood flow (it acts as a vasodilator). As a result, Xanthinol Nicotinate has been used to treat peripheral vascular disorders, arteriosclerotic conditions [2,3] cerebrovascular insufficiency [4], dementia [5] and hyperlipoproteinemia [6], etc. The absorption of two xanthinol nicotinate formulations has been reported by measuring plasma levels of nicotinic acid [7]. However, it is seemingly unreasonable to evaluate the bioavailability by measuring plasma levels of nicotinic acid because nicotinic acid is not the main active substance in xanthinol nicotinate formulation. In spite of a widespread use of the drug in medicinal products, only a method based on gas chromatography after derivatization with acetic anhydride had been reported to determine the concentrations of xanthinol in human plasma, in which the lower limit of quantification (LLOQ) was 2 μg/mL [8]. So, the development of a sensitive method for the determination of xanthinol in human plasma is of importance for the investigation of bioavailability and bioequivalence of this compound.

In this study, a simple, highly sensitive and selective LC–MS/MS method was developed and validated for quantitative analysis of xanthinol in human plasma and the established method was applied to the bioequivalence study in Chinese volunteers.

2. Experimental

2.1. Reagents and chemicals

Xanthinol nicotinate (purity > 99.9%, HPLC) and acyclovir (purity > 99.9%, HPLC) reference standard were purchased from National Institute for the Control of Pharmaceutical and Biological Products of China (Beijing, P.R. China). Structures of xanthinol nicotinate and acyclovir (internal standard, IS) are shown in Fig. 1. Methanol (HPLC grade) was obtained from TEDIA company, Inc. (Fairfield, OH, USA). Formic acid and trichloroacetic acid were

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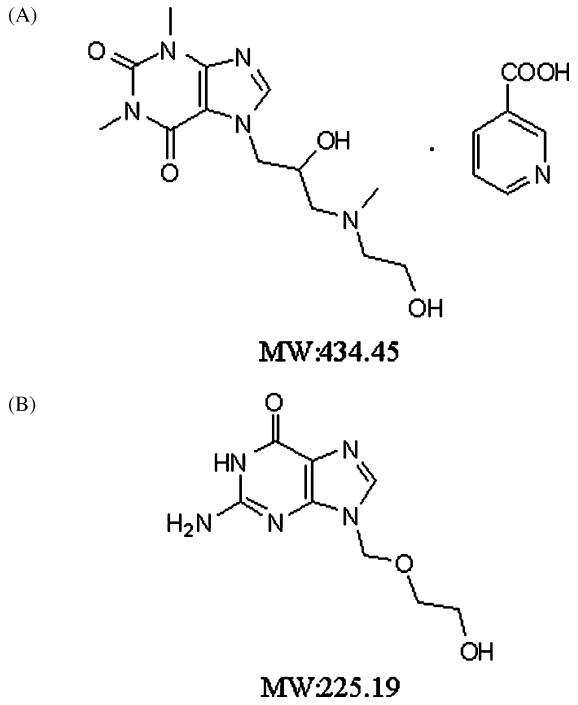

Fig. 1. Chemical structures for xanthinol nicotinate (A) and acyclovir (B).

of analytic-grade purity and purchased from Nanjing Chemical Reagent Co. Ltd. (Nanjing, P.R. China). Deionized water was purified through PL5242 Purelab Classic UV (PALL Co. Ltd., USA) before use. Blank plasma was supplied by Red Cross Society of China Nanjing Branch.

2.2. LC–MS/MS equipment

The LC–MS/MS system consisted of a Surveyor LC pump, a Surveyor auto-sampler and a TSQ Quantum Ultra AM triple–quadrupole tandem mass spectrometer with an electrospray ion source, Xcalibur 1.4 software for data acquisition and analysis (Thermo Finnigan, San Jose, CA, USA).

2.3. LC–MS/MS conditions

Chromatographic separations were performed using a Lichrospher silica (5 μm, 250 mm × 4.6 mm i.d., Hanban Science & Technology Co., Ltd., Huai'an, P.R.China) analytical column. The mobile phase of methanol–water containing 0.1% formic acid (50:50, v/v) was used isocratically eluting at a flow-rate of 1.0 mL/min. The total period for one sample was about 5.5 min. A 20 μL sample was injected into the column and 30% of the eluent was split into the inlet of the mass spectrometer using an electrospray ionization (ESI) source. The column temperature was maintained at 30 °C and the autosampler was set at 4 °C.

The mass spectrometer was operated in the positive ion detection mode with the spray voltage set at 5000 V. The heated capillary temperature was 350 °C. The nitrogen sheath gas and the auxiliary gas were set at 30 and 5 psi, respectively. Quantification was performed with selected reaction monitoring (SRM) with argon at a pressure of 1.0 mTorr for collision induced dissociation (CID) of the following transitions: xanthinol m/z 312.1 → 312.1 with the collision energy set at 10 eV, and acyclovir m/z 226.0 → 151.9 with collision energy set at 23 eV and a dwell time of 0.50 s per transition. The positive parent ion mass spectrum and product ion mass spectrum of xanthinol and acyclovir were shown in Figs. 2 and 3, respectively.

2.4. Analytical procedure

2.4.1. Preparation of stock solutions, calibration standard and quality control samples

A stock solution of xanthinol nicotinate in methanol at concentration of 123.2 μg/mL (based on xanthinol) was prepared. The internal standard (acyclovir) was also prepared as a stock solution (201.8 μg/mL) in methanol and was further diluted with methanol to 30.27 μg/mL and used for all analyses. A serial calibration curve

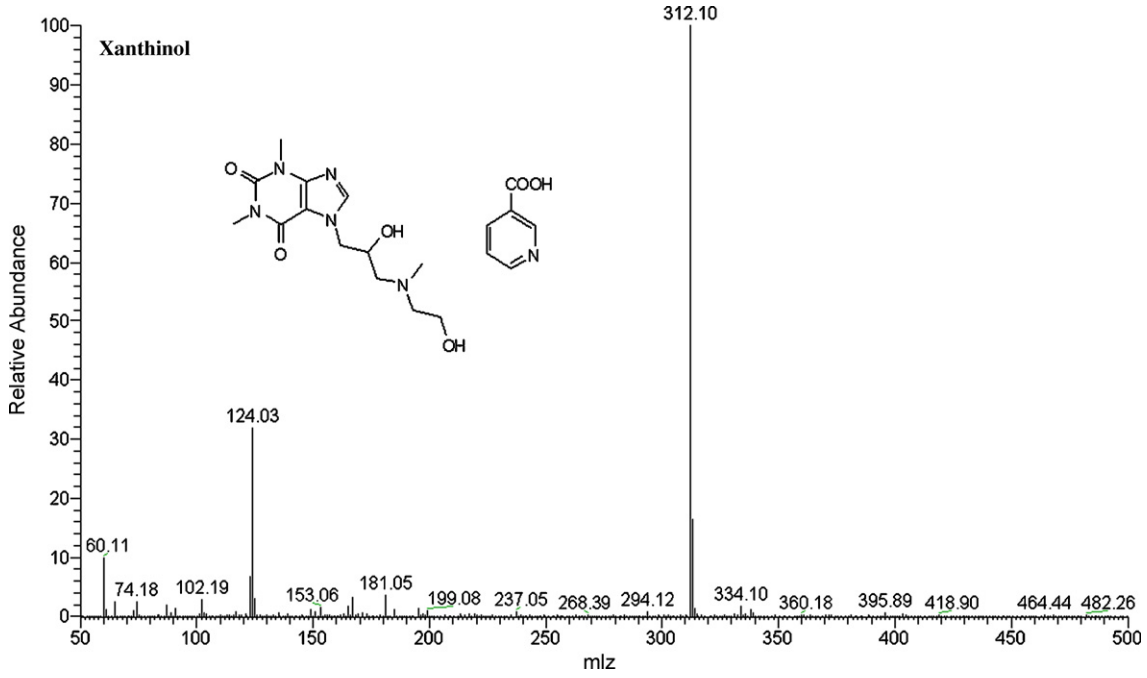

Fig. 2. Parent and product ion scan mass spectra of xanthinol (parent ion: m/z 312.1 $[M+H]^+$).

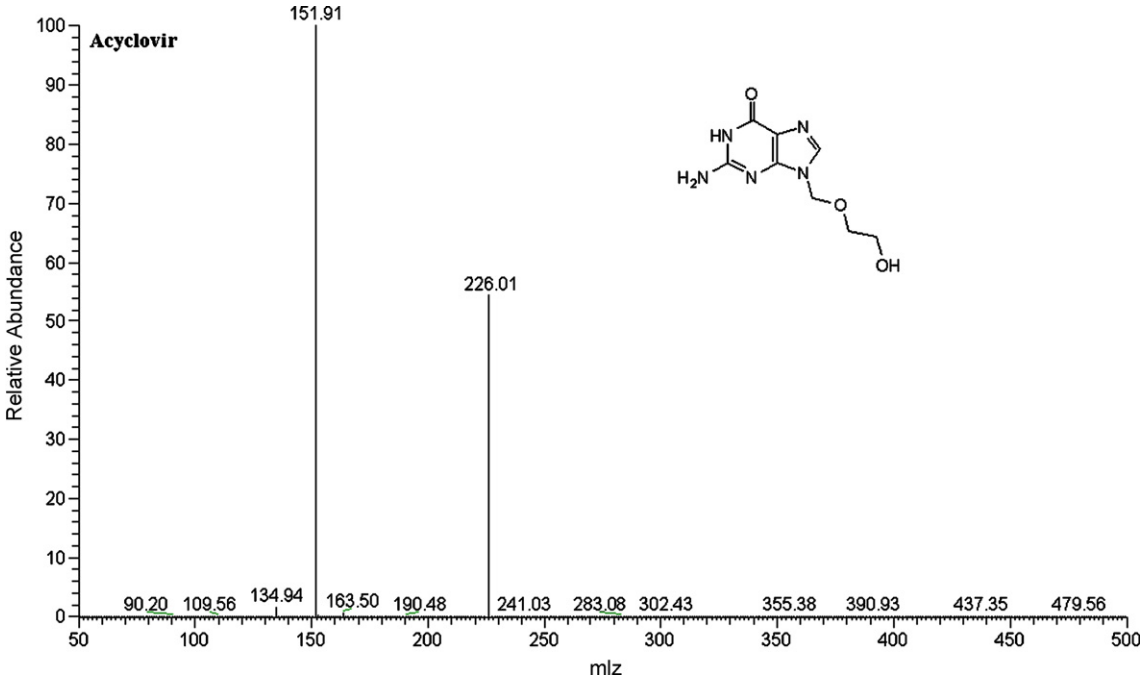

Fig. 3. Parent and product ion scan mass spectra of acyclovir (parent ion: *m/z* 226.0 $[M+H]^+$; product ion 151.9 *m/z*, collision energy: 23 eV).

samples at concentrations of 10.27, 20.54, 102.7, 205.4, 410.7, 821.4 and 1642.8 ng/mL (based on xanthinol) were freshly prepared by serially diluting stock solution with drug-free plasma. Firstly, plasma sample containing 1642.8 ng/mL of xanthinol was prepared by spiking with xanthinol nicotinate stock solution to drug-free plasma. Then the prepared plasma sample containing 1642.8 ng/mL of xanthinol was serially diluted with drug-free plasma to form calibration samples. The quality control (QC) samples were separately prepared by adding standard solution to drug-free plasma at concentrations of 10.27 (LLOQ), 20.54 (low), 205.4 (medium) and 821.4 ng/mL (high) of xanthinol. All plasma samples were stored at −20 °C. A 20 μL of IS (30.27 μg/mL) was added to 0.5 mL of calibration curve samples and QC samples, respectively. Further processing of both calibration curve samples and QC samples was as described in Section 2.4.2 for collection and preparation of the samples. All standard stock solutions were stored at −20 °C.

2.4.2. Collection and preparation of the samples

A 2 × 2, crossover, randomized, open-label study was conducted in Phase I Clinical Research Institute of the Jiangsu Province Hospital (Nanjing, P.R. China). The clinical trial protocol was approved by the Independent Ethics Committee (IEC) of Jiangsu Province Hospital. Twenty healthy male volunteers received the investigation. The average age of volunteers was 24.6 years old within the range of 22–27. The mean of body weights was 65 kg (59–70 kg) and the mean of body heights was 173 cm (167–180 cm). Subjects were included based on their medical history, clinical examination results and routine laboratory test results. All eligible subjects provided written informed consent for participation in the study. Subjects were randomly assigned to receive reference formulation followed by test formulation with a 2-week wash out period between doses. After a 12-h (overnight) fast, subjects received a single, 150-mg oral dose (tablet) of xanthinol nicotinate with 200 mL of water. Blood samples were collected in heparinized tubes predose (0 h) and at 0.33, 1, 1.5, 2.5, 3, 3.5, 4, 5, 6, 8, 10, 12, 15 and 24 h post-dose. Plasma was immediately separated by centrifugation at 4000 rpm and stored at −20 °C until analysis. A plasma sample (0.5 mL) was placed in a 1.5 mL Eppendorf tube. After the addition of 20 μL of 30.27 μg/mL solution of IS, the tube was briefly vortexed and 0.3 mL of 20% trichloroacetic acid was added into the tube. After vortexing for 3 min, the tube was centrifuged at 12,000 rpm for 10 min at room temperature, and then 20 μL of supernatant was injected onto the analytical column.

2.5. Assay validation

2.5.1. Selectivity

The selectivity was investigated by preparing and analyzing six individual human blank plasma and plasma samples at the LLOQ. Each blank plasma sample was tested using the protein precipitation procedure and LC–MS/MS conditions to ensure no interference of xanthinol and IS from plasma. For all plasma samples analyzed, there should be no peaks with single strength of >20% of the LLOQ for the analyte and no signal near the retention time of the IS.

2.5.2. Linearity of calibration curves and lower limit of quantification

Calibration standards of seven xanthinol concentration levels at 10.27, 20.54, 102.7, 205.4, 410.7, 821.4 and 1642.8 ng/mL were extracted and assayed. To evaluate the linearity, calibration curves were prepared and assayed on five days. The calibration curve was constructed by plotting the peak-area ratios of xanthinol to the IS versus the concentrations of xanthinol, using weighted least squares linear regression (weighting factor was $1/C^2$). The LLOQ was defined as the lowest concentration on the calibration curve at which precision was within 20% and accuracy was within ±20% [9], and it was established using five samples independent of standards. The QC samples were assayed along with clinical samples to monitor the performance of the assay and to assess the integrity and validity of the result of the unknown clinical samples analyzed.

2.5.3. Precision and accuracy

The validation samples were prepared and analyzed on three different days (one batch per day) to evaluate the accuracy, intra-

batch and inter-batch precision of the analytical method. The accuracy, intra-batch and inter-batch precisions of the method were determined by analyzing five replicates at 10.27, 20.54, 205.4 and 821.4 ng/mL of xanthinol along with one calibration curve on each of three batches. Assay precision was calculated using the relative standard deviation (R.S.D.%). The accuracy of an analytical method describes the closeness of mean test results obtained by the method to the true value of the analyte [9]. Accuracy is defined as the relative deviation in the calculated value (E) of a standard from that of its true value (T) expressed as a percentage (R.E.%). It was calculated using the formula: R.E.% = $(E - T)/T \times 100\%$.

2.5.4. Extraction recovery

The extraction recovery of xanthinol was evaluated by analyzing five replicates at 10.27 20.54, 205.4 and 821.4 ng/mL of xanthinol. The recovery was calculated by comparison of the peak areas of xanthinol extracted from plasma samples with those of injected standards.

2.5.5. Stability

The stability of xanthinol in plasma was studied under a variety of storage and handling conditions using the 10.27(LLOQ), 20.54 (low), 205.4 (medium) and 821.4 ng/mL (high) QC samples. The short-term temperature stability was assessed by analyzing QC samples that were kept at ambient temperature for 5 h. The stability of samples in autosampler was conducted reanalyzing extracted QC samples kept under the autosampler conditions (4 °C) for 8 h. Freeze–thaw stability (−20 °C in plasma) was checked through three cycles. The QC samples were stored at −20 °C for 24 h and thawed unassisted at room temperature. When completely thawed, the samples were refrozen for 24 h under the same conditions and thawed unassisted at room temperature. The freeze–thaw cycles were repeated three times, and then analyzed on the third cycle. The long-term stability was performed at −20 °C in plasma for 30 days.

2.5.6. Matrix effects

The matrix effect was measured by comparing the peak response of the post-extracted spiked sample with that of the unextracted sample containing equivalent amounts of the analytes of interest [10]. In set 1, the analytes were resolved in the blank plasma, and the obtained peak areas of the analytes were defined as A. In set 2, the analytes were resolved in mobile phase, and the obtained peak areas of the analytes were defined as B. ME was calculated by using the formula: ME (%) = $A/B \times 100$. The matrix effect of the assay was evaluated at four xanthinol concentration levels of 10.27, 20.54, 205.4 and 821.4 ng/mL and the IS concentration level of 30.27 μg/mL. Five samples at each level of the analytes were analyzed. The blank plasma samples used in this study were five different batches of human blank plasma. If the ME values exceed the range of 85–115%, an exogenous matrix effect is implied.

2.6. Pharmacokinetics

The maximum plasma concentrations (Cmax) and their time of occurrence (Tmax) of xanthinol were obtained directly from the observed data. The area under the plasma concentration–time curve (AUC) from the time zero to the last measured concentration (AUC_{0-24}) was calculated according to the linear trapezoidal rule. The terminal elimination rate constant (ke) was calculated by least-square regression of the plot of logarithms of concentration against time for the last five measurable points, the terminal half-life was calculated with $t_{1/2} = 0.693/ke$ accordingly, and the AUC_{0-24} was the corresponding area extrapolated to infinity by $AUC_{0-24} + C_{24}/ke$, where C_{24} was the plasma concentration of xanthinol at 24 h post-dose.

3. Results and discussion

3.1. Selection of internal standard (IS)

As a proper internal standard, it should be structurally or chemically similar to the analyte. And it should also have similar retention to the analyte, be well resolved from the analyte and other peaks, and mimic the analyte in any sample preparation steps [11]. Acyclovir was chosen as the internal standard for the assay because of its similarity of structure, retention and ESI ionization conditions to the analyte.

3.2. Sample preparation

In terms of sample preparation, protein precipitation was chosen because the extraction recovery was high, and the simple preparation procedure can save considerable time and simplify the operating process. Three kinds of precipitation reagents (trichloroacetic acid, acetonitrile and methanol) were tested as the protein precipitation reagents. The test results showed that methanol caused serious interference to the IS, and acetonitrile gave low recoveries of the analytes. Different concentrations of trichloroacetic acid were evaluated for the efficiency of protein precipitation; it was found that 20% trichloroacetic acid could precipitate the plasma proteins completely. So, 20% trichloroacetic acid was finally chosen as the precipitation reagent in the experiment.

3.3. Conditions for ESI-MS/MS

In order to develop a method with desired sensitivity (10 ng/mL), MS/MS detection was used instead of UV detection. Another benefit of using MS/MS detection is its inherent selectivity. Xanthinol was directly introduced into the MS detector using ESI ionization. Operation parameters such as the sheath gas, auxiliary gas, CID and collision energy were adjusted to increase the detection sensitivity of xanthinol. The optimum MS conditions are listed in Section 2.3.

Xanthinol and IS were separately scanned under the Q1 MS full scan mode to determine the parent ion, and under the Q1/Q3 (MS/MS) product ion scan mode to locate the most abundant production. $[M+H]^+$ was the predominant ion in the Q1 spectrum, and was used as the parent ion to obtain the product ion spectra. The most sensitive mass transition was from m/z 312.1libration curve samples at concentration312.1 for xanthinol with the collision energy set at 10 eV and from m/z 226.0libration curve samples at concentration151.9 for acyclovir (IS) with collision energy set at 23 eV, respectively. Considering the instability of product ion of xanthinol, m/z 312.1libration curve samples at concentration312.1 for xanthinol was chosen.

3.4. Conditions of HPLC

Different types of column (Lichrospher C18, Lichrospher CN and Lichrospher silica) were evaluated and the Lichrospher silica (5 μm, 250 mm × 4.6 mm i.d.) gave the best chromatography with a flow rate of 1 mL/min. The chromatographic conditions, especially by the composition of the mobile phase, were optimized through several trials to achieve good resolution and symmetric peak shapes of xanthinol and acyclovir. The experimental results showed that acidifying the aqueous portion of the mobile phase with formic acid could not only improve peak shapes of xanthinol and acyclovir, but

also increase the MS sensitivity. Different concentrations of formic acid at levels of 0.05%, 0.1% and 0.2% were tested in the aqueous portion of the mobile phase.

3.5. Assay validation

3.5.1. Selectivity

The selectivity was investigated by preparing and analyzing six individual human blank plasma and plasma samples at the LLOQ. Representative chromatograms of blank human plasma, the LLOQ (10.27 ng/mL based on xanthinol) of xanthinol nicotinate with IS in plasma and volunteer's plasma sample are shown in Fig. 4. Good selectivities for the analytes are shown by symmetrical resolution of the peaks, with no significant chromatographic interference nearby the retention times of the analytes and IS in the blank human plasma. Typical retention times for xanthinol and IS were 4.5 ± 0.05 and 3.9 ± 0.05 min, respectively. The total run time was about 5.5 min. Blank human plasma samples collected from 6 subjects were run up to 10 min. Because of the high seletivtiy of the SRM mode, no late-eluting interfering peaks were observed up to 10 min. In addition, for all plasma samples analyzed, there were no peaks with single strength of >20% of the LLOQ for the analyte and there were no signal nearby the retention time of the IS.

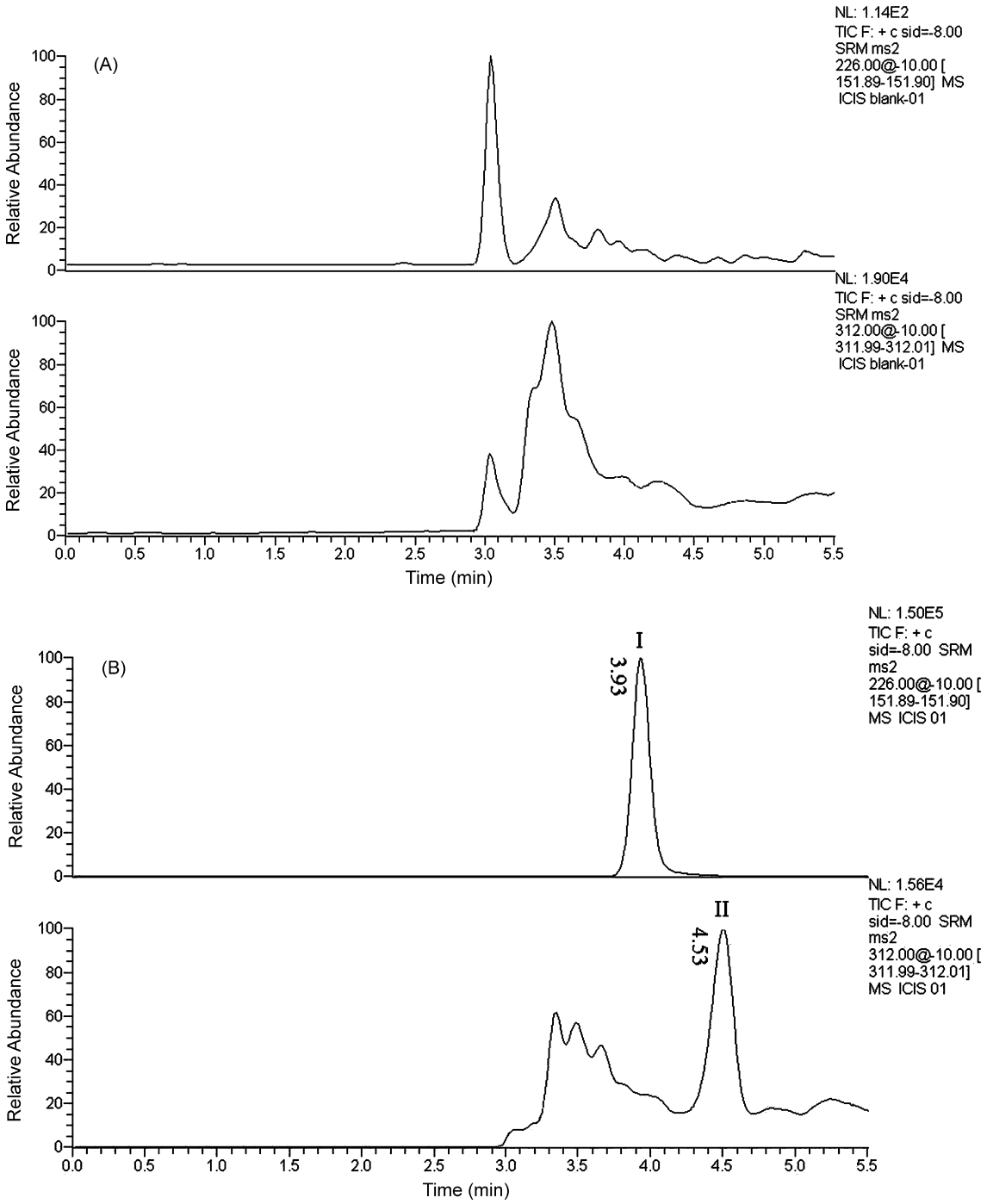

Fig. 4. Chromatograms by selected reaction monitoring (SRM) scan mode: (A) blank plasma (drug and IS free); (B) blank plasma spiked with 10.27 ng/mL (LLOQ) xanthinol and IS; (C) plasma sample of a subject 2.5 h post-oral administration of an xanthinol nicotinate tablet containing 107.5 mg xanthinol. Peak I: IS; Peak II: xanthinol.

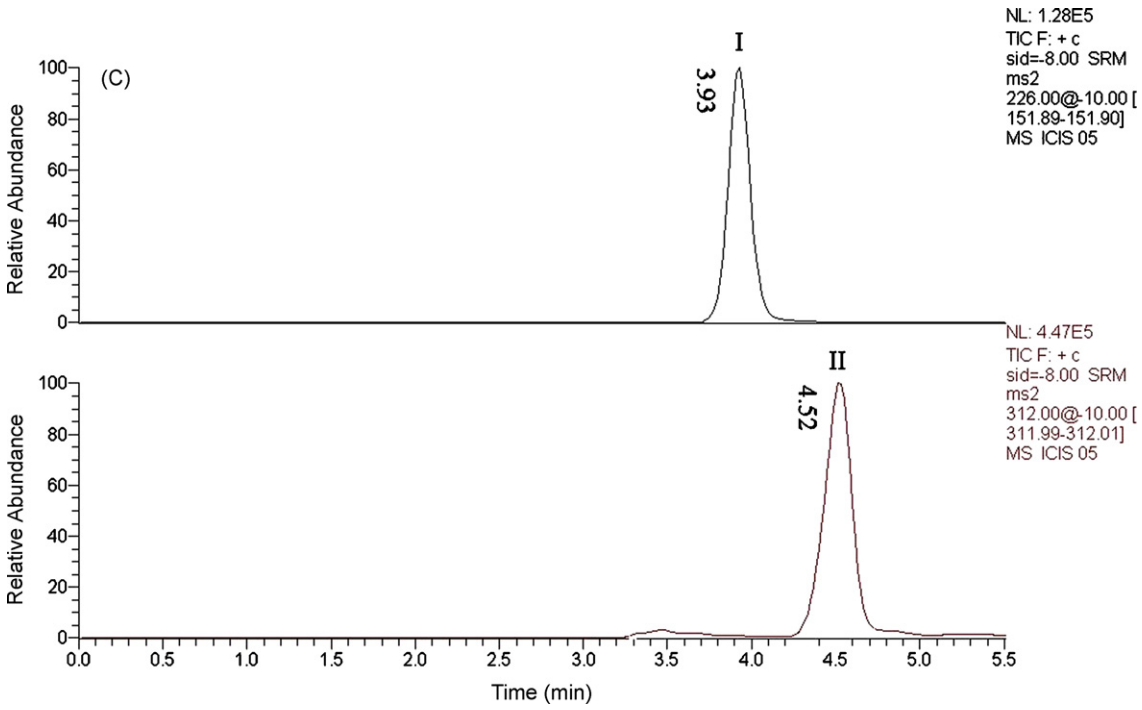

Fig. 4. (*Continued*).

Table 1
Results of five representative stand curves for xanthinol HPLC–ESI-MS determination

	Standard (ng/mL)							Standard curve results		
	STD1	STD2	STD3	STD4	STD5	STD6	STD7	Slope	Intercept	*r*
	10.27	20.54	102.7	205.4	410.7	821.4	1642.8			
	Found concentration (ng/mL)									
	10.08	21.19	101.1	229.1	393.1	777.7	1581	93.32	−0.3142	0.9978
	9.750	23.40	110.0	217.2	368.4	824.5	1405	86.61	−0.1051	0.9935
	10.06	20.75	116.2	235.0	360.4	721.2	1479	86.54	−0.8073	0.9917
	8.923	18.79	89.0	231.7	421.7	817.5	1508	92.73	−0.7346	0.9957
	10.22	20.68	100.9	217.9	415.8	817.7	1543	91.21	−0.5550	0.9992
Mean	9.807	20.96	103.4	226.2	391.9	791.7	1503	90.08	−0.5032	0.9956
S.D.	0.52	1.65	10.34	8.15	27.41	43.54	66.87	3.29	0.29	0.0031
R.S.D.%	5.3	7.9	10.0	3.6	7.0	5.5	4.4	3.7	NA	0.3
R.E.%	−2.7	−1.1	2.2	−1.4	−0.3	1.7	−4.7			

Note: Calibration curves were weighted $1/C^2$. STD, standard; R.S.D., relative standard; R.E., relative error; NA, not applicable.

3.5.2. Calibration curves and LLOQ

Five calibration analyses were performed on five days and the back-calculated values for each level were recorded (see Table 1). The calibration curves did not exhibit any nonlinearity within the chosen range (10.27–1642.8 ng/mL). The back-calculated results showed good day-to-day accuracy and precision.

The LLOQ for xanthinol in plasma was 10.27 ng/mL. The data of LLOQ obtained from six independent subjects are shown in Table 2. Acceptable mean accuracy of -3.9% (with %CV of 4.2%, $n = 6$) was obtained. This LLOQ level was selected because the concentrations of xanthinol in the samples from the bioequivalence study were expected to be close to it.

3.5.3. Assay precision and accuracy

Table 3 summarizes the intra- and inter-batch precision and accuracy for xanthinol evaluated by assaying the QC samples. The precision was calculated by using one-way ANOVA. In this assay, the intra-batch precision was 4.8% or less, and the inter-batch precision was 7.9% or less for each QC level of xanthinol. The results above demonstrate that the values are within the acceptable range and the method is accurate and precise.

3.5.4. Recovery

The choice of trichloroacetic acid as the extraction solvent may not only eliminate the interference of endogenous substances,

Table 2
Accuracy and precision for the analysis of LLOQ plasma samples obtained from six independent subjects ($n = 6$).

Concentration level (ng/mL)	Calculated concentration (ng/mL)	Mean (ng/mL)	R.S.D. (%)	R.E. (%)
10.27	10.13			−1.4
10.27	9.750			−5.1
10.27	10.05	9.933	5.3	−2.1
10.27	9.235			−10.1
10.27	10.21			−0.6
10.27	10.22			−0.5

Note: R.S.D., relative standard; R.E., relative error; n, number of replicates.

Table 3
Accuracy and precision for the analysis of xanthinol (in pre-study validation, three batches, five replicates per batch)

Parameter	Concentration (ng/mL)			
	10.27	20.54	205.4	821.4
Batch 1 (mean ± S.D.)	9.830 ± 0.46	22.25 ± 1.96	215.7 ± 21.95	781.6 ± 22.56
Batch 2 (mean ± S.D.)	9.980 ± 0.36	21.31 ± 0.94	215.8 ± 4.93	730.8 ± 32.85
Batch 3 (mean ± S.D.)	9.724 ± 0.47	19.63 ± 0.79	204.9 ± 2.92	729.8 ± 6.28
Overall mean	9.848	21.06	212.1	747.4
Intra-batch R.S.D. (%)	4.8	4.0	1.4	0.9
Inter-batch R.S.D. (%)	4.2	7.9	6.2	4.4
Overall accuracy (R.E.%)	4.1	−4.4	−0.2	−11.1

Note: S.D., standard deviation; R.S.D., relative standard; R.E., relative error.

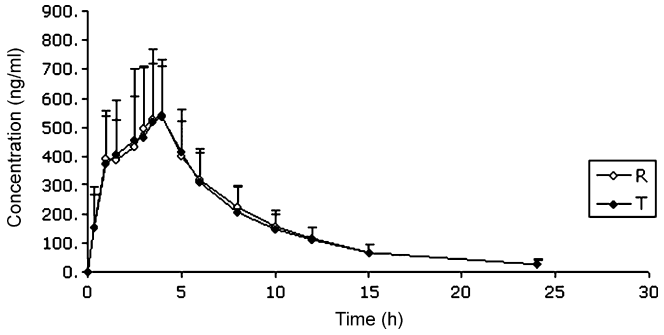

Fig. 5. Mean plasma concentration–time profile of xanthinol from 20 healthy volunteers following a single oral dose of 150 mg. T—test formulation; R—reference formulation.

but also meet the requirement of sensitivity for the assay. The recovery of xanthinol, determined at four concentration levels of 10.27, 20.54, 205.4 and 821.4 ng/mL were 89.9 ± 5.9%, 90.9 ± 7.9%, 96.41 ± 9.8% and 100.2 ± 2.9% (n = 5), respectively.

3.5.5. Stability

The stability of xanthinol in plasma was evaluated by analyzing quality control samples containing 20.54, 205.4 and 821.4 ng/mL of xanthinol after storage at ambient temperature for 5 h, after three freeze/thaw cycles, after storage in the autosampler at 4 °C, and after at storage −20 °C for 30 days. The mean percentages of deviation of calculated versus theoretical concentrations were less than or equal to 11% for short-term stability, less than or equal to 12.8% for freeze/thaw stability, less than or equal to 9.7% for autosampler stability, and less than or equal to 12.7% for long-term stability. The good stability of xanthinol simplified the precautions needed for laboratory manipulations during the analytical procedures.

3.5.6. Matrix effects

Matrix effect is generally a significant problem in the LC–MS/MS analysis of biological samples, in this assay, the ratios of the peak responses were 90.09 ± 2.3%, 102.1 ± 2.3%, 99.4 ± 1.8% and 103.9 ± 4.8% at 10.27, 20.54, 205.4 and 821.4 ng/mL concentrations for xanthinol and 100.9 ± 2.0% for I.S. respectively, indicating no coeluting endogenous substances interfering with the ionization of xanthinol or I.S. under the conditions of the assay.

3.6. Bioequivalence study

The method described above was successfully applied to the bioequivalence study in which plasma concentrations of xanthinol in 20 healthy male volunteers were determined up to 24 h after the administration of an oral dose of 150 mg xanthinol nicotinate. The plasma concentration maximum (Cmax) of test and reference formulations were 653 ± 238 ng/mL at 3.25 h and 615 ± 192 ng/mL at 3.38 h, the plasma elimination half-life ($t_{1/2}$) were 5.56 ± 2.26 h and 4.89 ± 1.42 h, the AUC_{0-24} were 4239 ± 1377 ng h/mL and 4287 ± 1166 ng h/mL and the $AUC_{0-\infty}$ were 4458 ± 1357 ng h/mL and 4457 ± 1205 ng h/mL, respectively. In this study in healthy volunteers, a single, 150-mg dose of test formulation was found to be bioequivalent to reference formulation based on the rate and extent of absorption. Representative concentration versus time profile for a subject, receiving a single dose, is presented in Fig. 5.

4. Conclusion

A sensitive, rapid and specific LC–MS method has been described for the determination of xanthinol in human plasma. No significant interferences caused by endogenous compounds were observed. The method has been successfully applied to the bioequivalence studies and the method has demonstrated to be reproducible. The pharmacokinetic parameters after a single 150-mg dose of xanthinol nicotinate in healthy volunteers were obtained.

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