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The integrated analysis strategy of unstable hypoxanthine, a potential quality marker in Shuxuetong injection based on standard addition method and multi-level pharmacokinetics by LC-MS/MS

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Abstract

Objective: As an injection made from traditional Chinese medicine, Shuxuetong (SXT) injection is used for the treatment of ischemic stroke. Hypoxanthine is regarded as one of its potential quality markers. The purpose of this study is to lay the foundation for the quality control of SXT injection by the analysis of the quantitation and pharmacokinetic behavior of hypoxanthine.

Methods: A quantitative method of hypoxanthine in SXT injection based on standard addition method by liquid chromatography-tandem mass spectrometry (LC-MS/MS) was established for the first time. On the other hand, a determination method of hypoxanthine in rat plasma samples after administration of SXT was also successfully established based on LC-MS/MS.

Results: It was found that the content of hypoxanthine was higher using conventional liquid-mass spectrometry technology compared to the application of LC-MS/MS combined standard addition method in the same batch of SXT injection. The ratio of low, medium and high doses of intravenous SXT were 1:2:4, and the AUC_{0-t} was (848.34 ± 324.53) μg·h/L, (1483.94 ± 497.74) μg·h/L, and (3074.84 ± 910.29) μg·h/L, respectively. AUC_{0-t} shows a good linear dose-dependent relationship.

Conclusions: The influences of endogenous substances tend to be eliminated by calibrating the concentration level of the target compound by the introduction of the standard addition method. The added allopurinol could inhibit the conversion of the target compound, and ensure the accuracy of the detection during the pharmacokinetic studies. "Blank biological matrix" obtained from the pretreatment of blank plasma successfully distinguished endogenous and drug-derived hypoxanthine. There is a good linear relationship between the blood concentration of intravenous hypoxanthine and the dosage of administration. Similarly, there was no drug accumulation in the multiple medium-dosage group, which is similar to the pharmacokinetic characteristics of the single medium-dosage group.

Keywords: Hypoxanthine, LC-MS/MS, Pharmacokinetics, Stability, Standard addition method, SXT injection

Graphical abstract: http://links.lww.com/AHM/A59.

Introduction

Shuxuetong (SXT) injection is an injection of traditional Chinese medicine (TCM), which is prepared by freezing-thawing extraction and membrane separation from *Whitmania pigra* Whitman (Shuizhi) and *Pheretima aspergillum* (E. Perrier) (Dilong). It has the effect of promoting blood circulation and removing blood stasis. Clinically, it is used in the acute phase of meridian in ischemic stroke caused by congestion, with syndromes containing hemiparalysis, deviation of eyes and mouth, language spasm, and acute cerebral infarction[1–2]. At present, the studies on SXT injection are mainly focused on evaluation and observation of pharmacological activity and clinical effects, and their results show that SXT has anticoagulation, thrombolytic, lipid regulation, and cell protection effects[3–5]. Chemical investigations have shown that both Shuizhi and Dilong contain amino acids, peptides, monosaccharides, polysaccharides, nucleosides, and nucleic acids[6–10]. However, studies on identifying responsible active compounds and their mechanisms in SXT injection are still limited.

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The material basis of SXT injection was studied in the early stage, and it was found that SXT injection contains a variety of nucleosides. Hypoxanthine is an intermediate metabolite in the pathway of purine metabolism *in vivo*, which are both present in Shuizhi and Dilong widely[11]. Therefore, in the compatibility environment, hypoxanthine is also the main component. A study has found that nucleosides including hypoxanthine have potential antithrombotic activity[12]. Moreover, hypoxanthine may be closely related to stroke rehabilitation at the same time[13]. In summary, it is speculated that hypoxanthine may be one of the quality markers (Q-markers) of SXT injection according to content distribution characteristics and pharmacological effects, which meets the principles of "effectiveness" "specificity" "transfer and traceability," "compatibility environment," and "measurability."

As we know, TCM includes plant medicine, animal medicine, and mineral medicine. It often contains a variety of endogenous ingredients, especially for animal drugs. These endogenous substances usually produce undesirable matrix effects when they were determined by LC-MS. To avoid the matrix effects, the matrix is often added to the standard curve solution. The matrix of biological samples such as plasma can be obtained through pre-processing (eg, the plasma matrix can be obtained by precipitating protein from blank plasma), while the standard calibration curve is prepared with pure solvents without the matrix in the determination process of the substances in TCM including animal drugs because blank matrix of TCM is quite difficult to obtain (it is hard to get rid of other substances except for the matrix)[14]. The result may be higher or lower than the true concentration level of samples using such a method. Therefore, in this article, we tend to develop a method based on the standard addition method to quantify the content of hypoxanthine in SXT injection, which means that the standard solution is added to the sample solution (the sample without pretreatment is regarded as a matrix). At the same time, the traditional LC-MS/MS method was used to determine the content of hypoxanthine in SXT injection, which was compared with the results of being combined with the standard addition method, to explore whether the introduction of the standard addition method can eliminate or reduce the influence by the complex impurities or matrix caused in SXT injection.

Hypoxanthine can be oxidized to xanthine and then to uric acid by xanthine oxidase (XO), which can also be directly oxidized to uric acid by XO (Supplementary Figure S1, http://links.lww.com/AHM/A58). The distribution and expression of XO in different species of animals as well as different organs, tissues, and blood are greatly different. There have been many reports on the determination of hypoxanthine in human blood and urine. However, due to the species differences of XO, the stability of hypoxanthine in rat's plasma remains to be confirmed[15–17]. Therefore, it is necessary to perform hypoxanthine stability experiments in rat plasma before performing pharmacokinetic experiments. It happens that there is a similar case, plant medicine also contains many unstable compounds that can be oxidized by oxygen, most of which have antioxidant activities. For example, echinacoside has not only low bioavailability but also poor stability. Vitamin C could inhibit the oxidation of echinacoside from the collection of plasma samples to ensure stability throughout the analysis process[18]. Likewise, allopurinol is a well-known specific inhibitor of XO[19–21]. In this study, we added allopurinol before collecting bio-samples. The results showed that allopurinol could effectively inhibit the activity of XO, block the metabolic pathway of hypoxanthine, and ensure the accurate quantification of hypoxanthine[22–23].

In this article, a study on the pharmacokinetics of hypoxanthine in SXT was designed to investigate on pharmacokinetic behavior of hypoxanthine in SXT. Accordingly, a single intravenous low dosage (1.62 mL/kg), medium dosage (3.24 mL/kg), high dosage (6.48 mL/kg), and multiple dosages (3.24 mL/kg) were designed to be used for the investigation of pharmacokinetic parameters of hypoxanthine in SXT injection.

Materials and methods

Chemicals and reagents

Methanol (chromatographic grade), acetonitrile (chromatographic grade), and hypoxanthine (purity ≥ 99%) were purchased from Sigma Aldrich (St. Louis, MO, USA). Distilled water was purchased from Hong Kong Watsons limited company. Formic acid was purchased from America TEDIA limited company. SXT injection was provided by Mudanjiang Youbo limited liability company. 6-mercaptopurine (purity ≥ 99%) and 6-chloropurine standards (purity ≥ 99%) were purchased from Sigma Aldrich. Hypoxanthine (purity ≥ 99%) and allopurinol (purity ≥ 98%) were purchased from Shanghai Yuan ye limited company.

Animals

Male Sprague-Dawley rats [weighing (200 ± 10) g] were bought from HFK Bioscience Co. Ltd. (Beijing, China). All the animals were given standard laboratory food and water. They were kept under the same breeding room conditions with temperature (20–25)°C and humidity controlled (40%–60%). The study followed the guidelines of the ethics committee of the Experimental Animal Center of Tianjin University of Traditional Chinese Medicine.

Sample preparation method

Preparation of stock and reference solutions

Preparation of stock solution: 0.74 mg of hypoxanthine was weighed and 100 μg/mL hypoxanthine methanol stock solution was prepared by adding methanol. Separately, 0.95 mg of 6-mercaptopurine was weighed, and a 6-mercaptopurine methanol stock solution was prepared as the concentration of 125 μg/mL.

Preparation of reference solution: dilute the hypoxanthine stock solution and the 6-mercaptopurine stock solution with formic acid (a) and methanol (b) (a:b, 1:500) to prepare a series of concentrations of hypoxanthine (containing an internal standard solution of 250 ng/mL). Their concentrations are 10, 20, 50, 100, 200, 500, 1,000, and 5,000 ng/mL.

Preparation of QC samples without plasma matrix: dilute the hypoxanthine stock solution and the 6-mercaptopurine stock solution with formic acid (a) and methanol (b) (a:b, 1:500) to prepare low, medium, and high concentrations of hypoxanthine (containing an internal standard solution of 250 ng/mL). The concentration of the three quality control samples is 20, 200, and 3,750 ng/mL.

Preparation of spiked samples

The 100 μL SXT injection was added to six 1.5 mL centrifuge tubes containing 100 μL of 6-mercaptopurine internal standard solution at a concentration of 125 ng/mL. And then, 0, 100, 200, 300, 400, 500 μL of 50 μg/mL hypoxanthine standard solution were added to these six centrifuge tubes in order. These samples were diluted to 1.00 mL with formic acid (a), methanol (b), water (c) (a:b:c, 1:500:2,000), and they were diluted 10 times with the same solvent. 200 μL of each sample was then taken and diluted 5 times with the same solvent, which was made to 0 ng, 100, 200, 300, 400, and 500 ng/mL of the spiked solutions containing 250 ng/mL internal standard using the same solvent.

Preparation for hypoxanthine plasma stability

Six experimental groups were used to evaluate the plasma stability of hypoxanthine, which are, respectively BP, BP + A, BP + SXT, BP + H, BP + SXT + A, and BP + H + A. The sample of BP was prepared by collecting whole blood from 3 randomly selected rats, mixing them uniformly and centrifuging at 8,000 r/min for 10 min. The blank plasma samples required in the remaining five groups refer to the above preparation protocol. BP + A is composed of blank plasma sample and allopurinol, BP + SXT is composed of 450 μL blank plasma sample and 50 μL SXT injection, BP + H is composed of 450 μL blank plasma sample and 50 μL hypoxanthine reference solution, BP + SXT + A is composed of 450 μL blank plasma sample, 50 μL SXT injection and allopurinol and BP + H + A is composed of 450 μL blank plasma sample, 50 μL hypoxanthine reference solution and allopurinol. The dried allopurinol is added to the centrifuge tube before adding other samples and heparin sodium is added to each sample for anticoagulation. The samples of BP and BP + A were placed at room temperature (25°C) for 0, 0.25, 0.5, 0.75, 1, 2, 4, and 6 h. The samples of BP + SXT, BP + H, BP + SXT + A and BP + H + A were placed at room temperature (25°C) for 0, 0.25, 0.5, 0.75, 1, 2, 24, and 48 h. Three parallel samples in which 50 μL samples were taken at each time point for sample determination.

Plasma sample pretreatment method

The 50 μL blank plasma, 50 μL 6-chloroprine (IS2) and 50 μL formic acid (a) and water (b) (a:b, 1:500) were added into the centrifuge tube. Three hundred fifty microliters of methanol was added to the centrifuge tube after the sample was swirled and mixed for 2 min and the sample was continued to be swirled for 5 min and centrifuged at 14,000 r/min for 10 min before taking 400 μL of the supernatant. Finally, the samples were centrifuged at 14,000 r/min for 5 min before determination.

Administration and blood collection method in rats

The experiment was divided into four groups (n = 10), including single intravenous low dosage (1.62 mL/kg), medium dosage (3.24 mL/kg), high dosage (6.48 mL/kg), and multiple intravenous medium dosages (3.24 mL/kg). The administration dosages of the low, medium, and high dosage groups were equivalent to 3, 6, and 12 times of the human dose, respectively. The rats were preconditioned for 1 week, and fasted for 12 h before the test. SXT injection (batch number: 17040421) was injected by the tail vein. Whole blood was collected at time points 0.083, 0.167, 0.333, 0.500, 0.750, 1, 2, 3, 4, 6, and 8 h after injection of SXT injection and then added into a centrifuge tube with heparin and allopurinol. After 8,000 r/min of centrifugation at 4°C for 10 min, the upper plasma was collected and stored in −70°C refrigerator. Rats in the group of multiple doses were given the medium dosage (3.24 mL/kg) for seven consecutive days. SXT injection was injected by the tail vein once a day and they were fed uniformly 4 h after intravenous injection. Whole blood was collected at time points 0.083, 0.167, 0.333, 0.500, 0.750, 1, 2, 3, 4, 6, and 8 h after injection of SXT injection on the last day, which was added into a centrifuge tube with heparin and allopurinol. The upper plasma was collected and stored in −70°C refrigerator after centrifugation at 8,000 r/min for 10 min.

Methods for determination of hypoxanthine

UPLC method

Determination method of hypoxanthine in SXT injection was as follows. Samples were detected on a Waters ACQUITY H-Class UPLC system connected to a Waters XEVO TQ-S triple quadrupole mass spectrometer (Milford, MA, USA). Chromatographic separation was achieved on a Waters ACQUITY UPLC BEH C_{18} (100 mm × 2.1 mm, 1.7 μm) maintained at 40°C. The mobile phase consisted of methanol (A) and water containing 0.1% formic acid (B). The gradient program was as follows: 0–0.5 min, 15% A-15% A; 0.5–0.6 min, 15% A-95% A; 0.6–1.0 min, 95% A-95% A; 1.0–1.1 min, 95% A-5% A; 1.1–3.5 min, 5% A-5% A. The flow rate was 0.3 mL/min and the injection volume was 2 μL. Sample chamber temperature was 10°C.

Determination method of hypoxanthine in plasma samples was as follows. Samples were detected on a Waters ACQUITY H-Class UPLC system connected to a Waters XEVO TQ-S triple quadrupole mass spectrometer. Chromatographic separation was achieved on an Agilent ZORBAX SB-C_{18} (4.6 mm × 100 mm, 1.8 μm) maintained at 40°C. The mobile phase consisted of methanol (A) and water containing 0.1% formic acid (B). The gradient program was as follows: 0–0.3 min, 15% A-15% A; 0.3–0.5 min, 15% A-40% A; 0.5–1.0 min, 40% A-70% A; 1.0–1.5 min, 70%

A-95% A; 1.5–2.0 min, 95% A-95% A; 2.0–2.01 min, 95% A-5% A; 2.01–3.5 min, 5% A-15% A. The flow rate was 0.8 mL/min and the injection volume was 2 µL. Sample chamber temperature was 10°C.

Mass spectrometry method

Determination method of hypoxanthine in SXT injection was as follows. The ESI source parameters were set as fellows: capillary voltage, 3.0 kV (ESI^-); source offset, 50 V; cone, 30 V; nebulizer, 7 psi; cone gas flow, 150 L/h; collision gas Flow, 0.15 mL/min; desolvation temperature, 400°C; and desolvation gas flow, 800 L/h (N_2, purity > 99.9%). Sensitive detection was achieved in multiple reaction monitoring (MRM) mode, which was in the negative ion mode; and the key parameters involving chemical transitions, cone voltage (CV), and collision energy (CE) are given in Table 1. Data acquisition and processing were carried out by using Mass Lynx 4.1 and Target Lynx (Waters), respectively.

Determination method of hypoxanthine in plasma samples was as follows. The ESI source parameters were set as fellows: capillary voltage, 2.0 kV (ESI^+); source offset, 50 V; cone, 30 V; Nebulizer, 7 psi; cone gas flow, 150 L/h collision gas Flow, 0.15 mL/min; desolvation temperature, 400°C; and desolvation gas flow, 800 L/h (N_2, purity > 99.9%). Sensitive detection was achieved in the MRM mode, which was in the positive ion mode; and the key parameters involving chemical transitions, CV, and CE are given in Table 2. Data acquisition and processing were carried out by using Mass Lynx 4.1 and Target Lynx (Waters), respectively.

Method validation

Following Related guides of the US Food and Drug Administration, the assay methods were validated concerning specificity, precision, accuracy, linearity and range, matrix effects and extraction recovery as well as stability[24]. The specific content of this section is shown in Supplemental Digital Content, http://links.lww.com/AHM/A58.

Table 1.

MRM parameters of the analytes in SXT injection

Compound	Q1	Q3	Dwell (ms)	Cone (V)	Collision (V)
Hypoxanthine	134.9	92.0	129	30	12
6-Mercaptopurine (IS1)	151.0	92.1	129	56	16

MRM: Multiple reaction monitoring; SXT: Shuxuetong.

Table 2.

MRM parameters of the analytes in plasma samples

Compound	Q1	Q3	Dwell (ms)	Cone (V)	Collision (V)
Hypoxanthine	136.88	110.13	162	42	20
6-chloroprine (IS2)	155.04	119.08	162	10	18

MRM: Multiple reaction monitoring.

Results

Method validation

Determination of hypoxanthine in SXT injection

The resolution and peak shape of hypoxanthine (target compound) and 6-mercaptopurine (IS1) are acceptable. The result of the specificity is shown in Figure 1. The intra-precision, inter-precision, and stability measured at three levels were below 1%. Recovery is close to 100%, while repeatability is within acceptable limits. The regression equation of hypoxanthine was $Y = 5.664 \times 10^{-3} X + 1.447$, $r = 0.9998$, and the linearity was ranged between 0 to 500 ng/mL. The LOQ was 0.3 ng/mL. The specific results (Tables S4–S6) are displayed in Supplemental Digital Content, http://links.lww.com/AHM/A58.

Determination of hypoxanthine in plasma samples

The resolution of hypoxanthine and 6-chloropurine (IS2) is acceptable. No significant interference was detected at the retention time. The result of the specificity is shown in Figure 2. Precision (intra and inter) measured at three levels was below 15%, and accuracy varied within the range of 85.0%–115.0%, which could indicate good precision and acceptable accuracy for the established assay. The regression equation of hypoxanthine was $Y = 2.31 \times 10^{-3} X + 3.29 \times 10^{-2}$ ($r = 0.9993$), and the linearity ranged between 10 and 5,000 ng/mL. The extraction recovery and matrix effect of hypoxanthine were both in the acceptable range. Hypoxanthine could remain stable within 12- and 24-hour short-term and long-term stabilities. The specific results (Tables S7–S9) are displayed in Supplemental Digital Content, http://links.lww.com/AHM/A58.

The content determination results of hypoxanthine in SXT injection of 20 batches

Twenty batches of SXT injection were prepared as samples according to the above sample preparative method. This method successfully detected the content of hypoxanthine in 20 batches of SXT injection, and the results showed that its concentration was uniformly good in these 20 batches. Hypoxanthine in those samples was detected using a standard addition method based on LC-MS/MS, and the results were compared with those of the general method without the introduction of the standard addition method (Figure 3). The content of hypoxanthine in 20 batches of SXT injection based on LC-MS/MS without the introduction of the standard addition method is shown in Supplemental Digital Content, http://links.lww.com/AHM/A58.

Hypoxanthine plasma stability

The average value of the stability of the obtained plasma sample was plotted into a line chart, with the abscissa as time and the ordinate as concentration, which is shown in Figure 4. Among them, the line chart of the results of BP and BP + A is shown in Figure 4A. The line chart of BP + H and BP +H+ A is shown in Figure 4B. The line chart of the results of BP + SXT and BP + SXT + A is shown in Figure 4C.

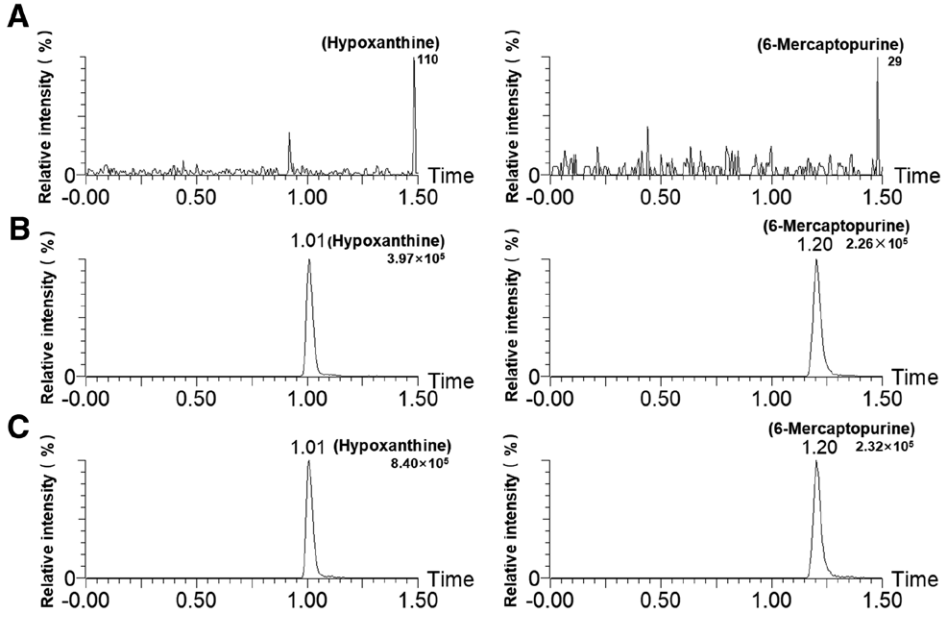

Figure 1. MRM chromatogram of hypoxanthine (target compound) and 6-mercaptopurine (IS1). (A) The MRM chromatogram of blank saline. (B) The MRM chromatogram of sample solution. (C) The MRM chromatogram of sample solution spiked with hypoxanthine standard. MRM: multiple reaction monitoring.

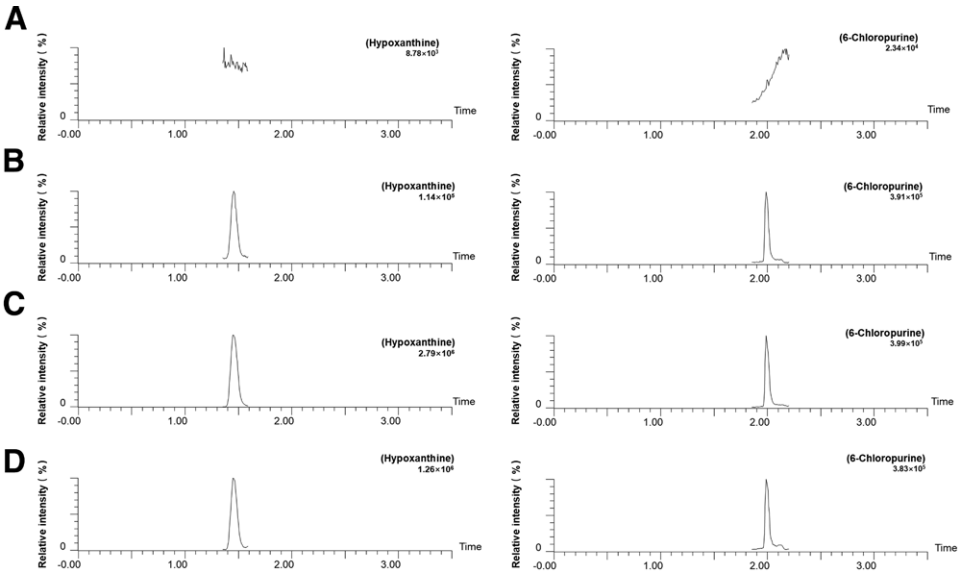

Figure 2. The MRM chromatogram of hypoxanthine (target compound) and 6-chloropurine (IS2): (A) The MRM chromatogram of blank plasma matrix without allopurinol (B) The MRM chromatogram of blank plasma matrix with allopurinol. (C) The MRM chromatogram of blank plasma matrix spiked with standard. (D) The MRM chromatogram of the rat plasma in 5 min after administration. MRM: multiple reaction monitoring.

From the results of Figure 4A, it can be known that the original XO in the plasma can be inactivated after the blank plasma is immediately pretreated to remove the protein, and the hypoxanthine content can be measured. Hypoxanthine content was significantly reduced after 0.25 h at room temperature. However, its content was almost stable when allopurinol was added. According to Figure 4B and C, hypoxanthine content of blank plasma spiked hypoxanthine standard solution and blank plasma spiked SXT injection gradually decreased with time at room temperature. Hypoxanthine of blank plasma spiked hypoxanthine standard solution with allopurinol and blank plasma spiked SXT injection with allopurinol can still be detected after 48 h at room temperature. Based on the above results, it is obvious that rapidly oxidative metabolism due to the purine oxidase would affect the quantification of hypoxanthine seriously, and allopurinol could effectively inhibit XO enzyme activity, and block the pathway of hypoxanthine metabolism (Supplementary Figure S2, http://links.lww.com/AHM/A58). Therefore, an appropriate amount of allopurinol was essentially added to the centrifuge tube before collecting whole blood from the intraocular canthus of the rat to ensure accurate quantification of hypoxanthine.

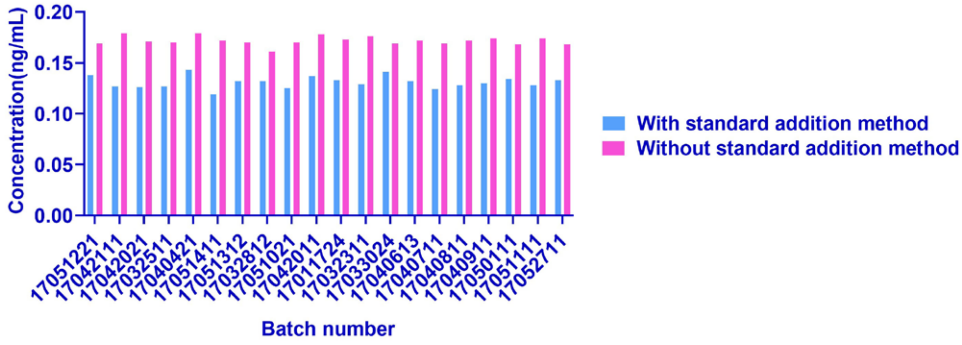

Figure 3. Histogram of the determination results of hypoxanthine in SXT injection of 20 batches using two different detection methods. SXT: Shuxuetong.

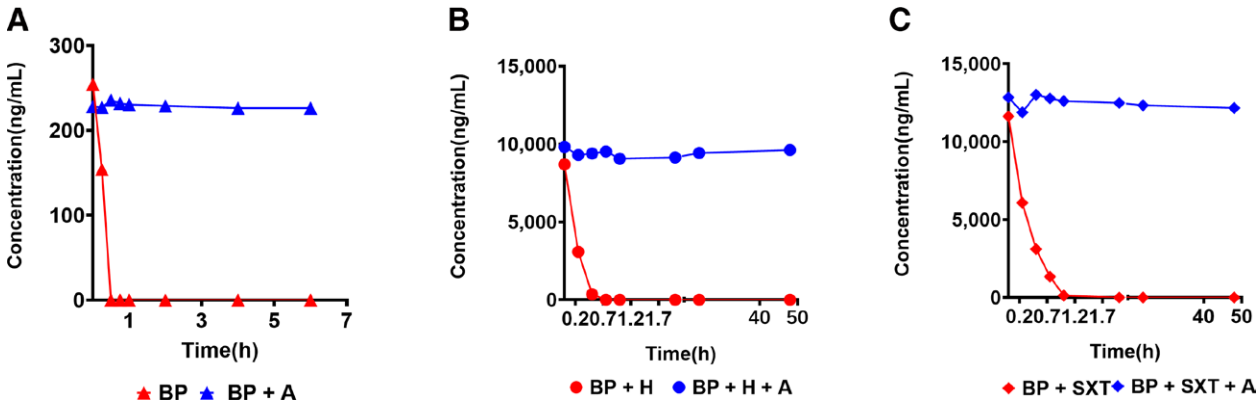

Figure 4. Line chart of hypoxanthine concentration in plasma samples over time.

Pharmacokinetic results of hypoxanthine in SXT injection

The validated quantitative method was successfully applied to investigate the pharmacokinetic behaviors of hypoxanthine in intravenous single low dosage (1.62 mL/kg), medium dosage (3.24 mL/kg), high dosage (6.48 mL/kg), and multiple dosage (3.24 mL/kg) groups. The plasma concentration–time curves are shown after intravenous administration of SXT injection from these four groups in Figure 5, while the corresponding PK parameters of hypoxanthine are listed in Table 3. The diagram of the relationship between $AUC_{0\text{-}t}$ and dose after a single administration of hypoxanthine is shown in Supplementary Figure S5, http://links.lww.com/AHM/A58. Blood was collected before the administration to determine the trough concentration on the fourth, fifth, and sixth days after continuous administration of the rats for the multiple intravenous doses (3.24 mL/kg) group. The histogram and box plot of blank concentration and trough concentration of multiple intravenous doses (3.24 mL/kg) group are shown in Figure 6.

Discussion

Since hypoxanthine is an endogenous component, distinguishing the substance to be tested from the rat or the exogenous drug is pivotal. In this study, the rat blank plasma was treated to remove endogenous hypoxanthine and obtain the true "empty" for methodology development and evaluation. Endogenous hypoxanthine was oxidized and metabolized completely by XO after rat plasma was placed at room temperature at least for 2 h. With this method applied, endogenous hypoxanthine can be removed to obtain a true "blank biological matrix."

Hypoxanthine was mostly detected using electrochemical analysis methods and HPLC in previous studies[25–29]. Although these methods are mature and simple, they are not suitable for complex samples at lower concentrations. Standard addition method is adding a certain amount of standard into the test sample, and determining the concentration before and after addition. It is particularly suitable for testing when there is the presence of complex matrix in a sample. Combining liquid chromatography-mass spectrometry and standard addition method, our method could be utilized for endogenous metabolites determination successfully[30–32].

As we all know, many types of TCM are derived from nature, which contain many endogenous compounds, especially animal Chinese medicines. These endogenous substances may produce matrix effects in the co-elution process with the target compound, the effects of which may enhance or inhibit the response of the target compound and are difficult to be found when a single analytical method is used[14]. It is found that the content of hypoxanthine is higher using conventional liquid-mass spectrometry technology than the application of LC-MS/MS combined with standard addition method in the same batch of SXT injection. Therefore,

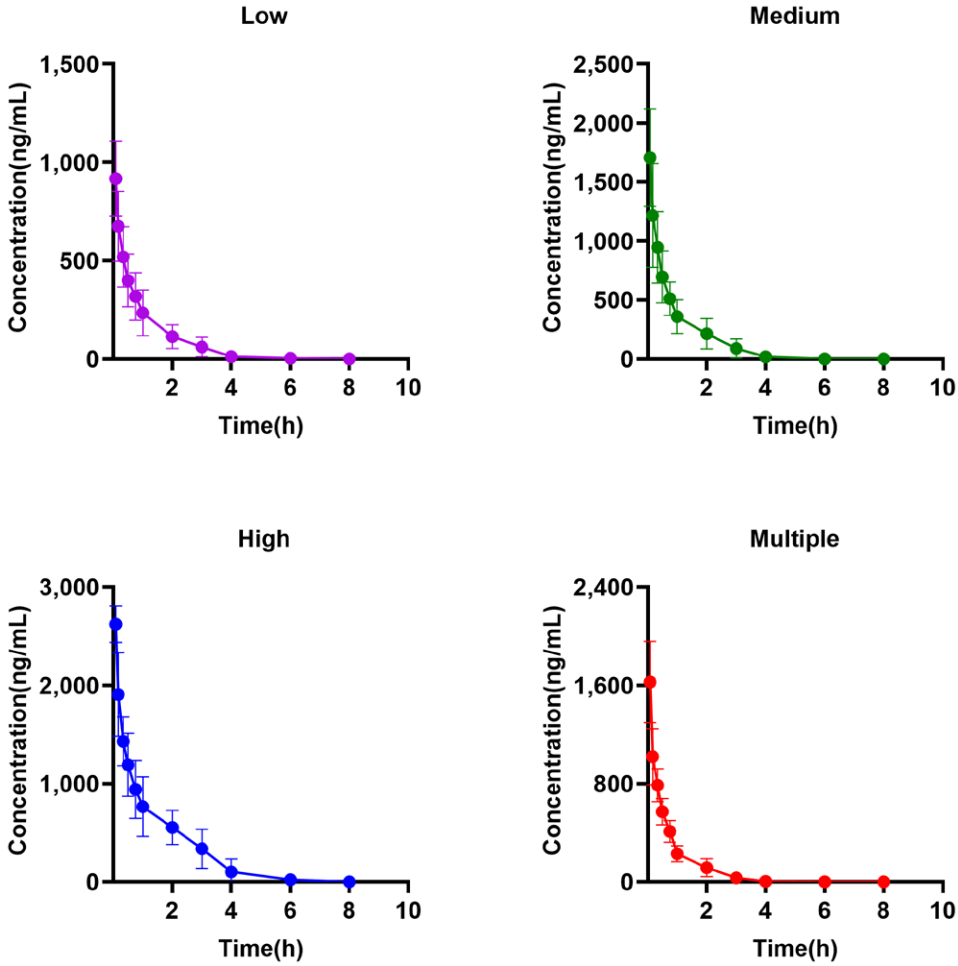

Figure 5. The plasma concentration–time curves of hypoxanthine.

Table 3.

Pharmacokinetic parameters of hypoxanthine.

Parameter	C_{max} (ng/mL)	T_{max} (h)	$t_{1/2}$ (h)	MRT (h)	CL (L/kg/h)	V_d (L/kg)	AUC $_{0\text{-}8\,h}$ (μg·h/L)	$AUC_{0\text{-}\infty}$ (μg·h/L)
Low	917.25 ± 189.41	0.08	0.61 ± 0.19	0.87 ± 0.27	26.50 ± 9.03	33.14 ± 13.51	782.03 ± 264.00	848.34 ± 324.53
Medium	1,707.64 ± 414.25	0.08	0.56 ± 0.14	0.80 ± 0.20	14.75 ± 4.33	21.18 ± 10.55	1,315.16 ± 445.33	1,483.94 ± 497.74
High	2,622.51 ± 186.73	0.08	0.81 ± 0.23	1.16 ± 0.34	7.00 ± 1.95	11.71 ± 4.17	2,793.81 ± 917.69	3,074.84 ± 910.29
Multiple	1,626.13 ± 330.69	0.08	0.42 ± 0.11	0.61 ± 0.16	19.97 ± 5.10	18.73 ± 4.84	987.25 ± 222.15	1,065.33 ± 283.39

C_{max} (maximum concentration), T_{max} (time to reach C_{max}), $t_{1/2}$ (elimination half-life), MRT (mean residence time), CL (plasma clearance), V_d (apparent volume of distribution), AUC $_{0\text{-}8h}$ (area under the plasma concentration-time curve from time 0 to 8h of quantifiable concentration), $AUC_{0\text{-}\infty}$ (area under the plasma concentration-time curve from time 0 to infinite time). Results are displayed as the mean ± standard deviation (SD).

other endogenous substances enhance the response value of the target compound in this experiment. Such results suggest that it is often unreliable to use a single analytical method to detect herbal medicines containing endogenous ingredients because it cannot reflect the true concentration of the target compound. However, the introduction of similar methods covering standard addition method, the influences of complex impurities tend to be eliminated to calibrate the concentration of the target compound.

Allopurinol, as an inhibitor of XO, can inhibit hypoxanthine conversion. It was added into the centrifuge tube before the experimental blood collection to ensure accurate determination of hypoxanthine in rat plasma. In this experiment, the total concentration of hypoxanthine minus the concentration of hypoxanthine in blank plasma was used as a corrected concentration as endogenous hypoxanthine is widely found in rats. Histogram of hypoxanthine concentration in rat blank plasma from four experimental groups is shown in Figure 7.

Rats were administrated with SXT injection in the tail vein, and the drug directly entered into the blood circulation. Plasma concentration of hypoxanthine was decreased with time due to rapid metabolism ($T_{1/2}$ < 0.8 h), which was related to the higher XO activity in rats. Hypoxanthine is rapidly and continuously oxidized to uric acid by XO in rats and uric acid is further oxidized to allantoin by urase. The ratio of low, medium, and high dosages of SXT was 1:2:4, and the $AUC_{0\text{-}t}$ was

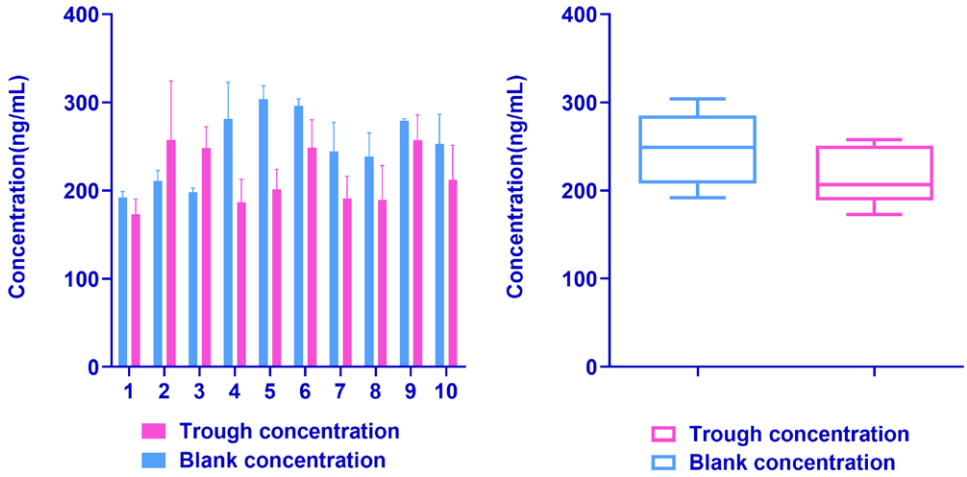

Figure 6. Histogram and box plot of blank concentration and trough concentration of multiple intravenous doses (3.24 mL/ kg) group.

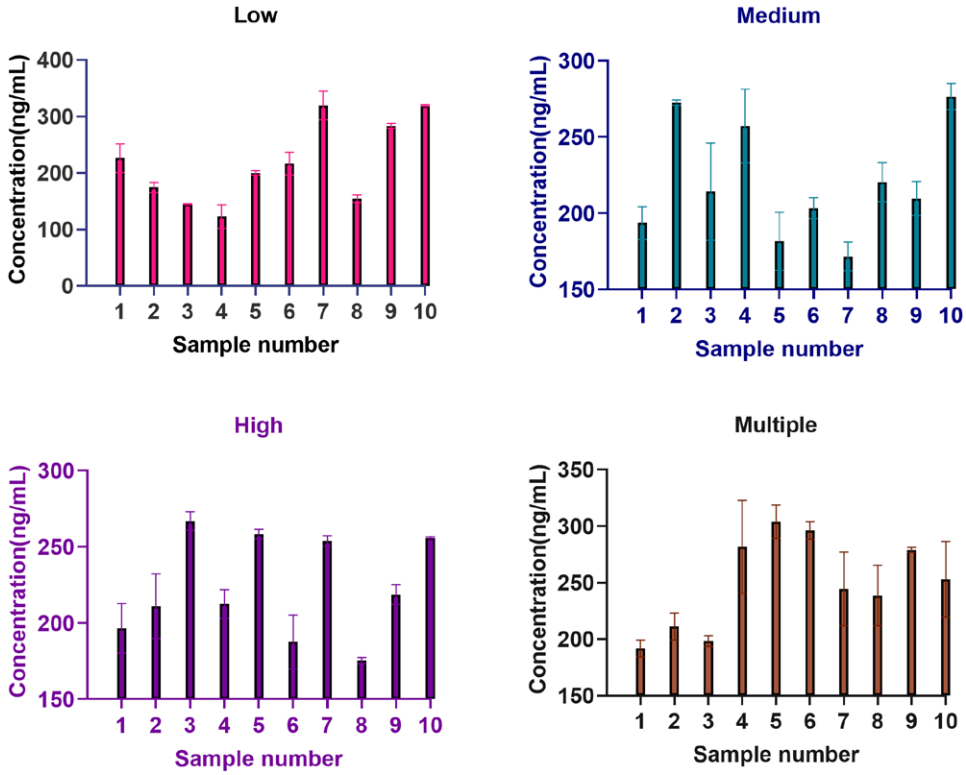

Figure 7. Histogram of hypoxanthine concentration in blank plasma of rats in the four different doses administered groups.

(848.34 ± 324.53) μg·h/L, (1,483.94 ± 497.74) μg·h/L, and (3,074.84 ± 910.29) μg·h/L, respectively. The results (in Figure 8) show that there is a good linear relationship between the blood concentration of intravenous hypoxanthine and the dosage. Results of determination of trough concentration in multiple intravenous dosages group and the endogenous hypoxanthine have no significant difference according to Figure 6. Therefore, there was no drug accumulation in the multiple medium-dosages group.

Conclusions

A quantitative method of hypoxanthine in SXT injection based on standard addition method by LC-MS/MS was established for the first time, which proved to be accurate and reliable. The method could exclude matrix effect caused by endogenous substances in the injection. On the other hand, a determination method of hypoxanthine in rat plasma samples was established by LC-MS/MS for the first time. The added allopurinol inhibited the conversion of the target compound and stabilized it, ensuring the accuracy of the detection during the pharmacokinetic study. "Blank biological matrix" obtained from pretreatment of blank plasma successfully distinguished the endogenous and drug-derived hypoxanthine. $AUC_{0\text{-}t}$ shows good linear dose-dependent relationships. Similarly, there was no drug accumulation in the multiple medium-dosages group.

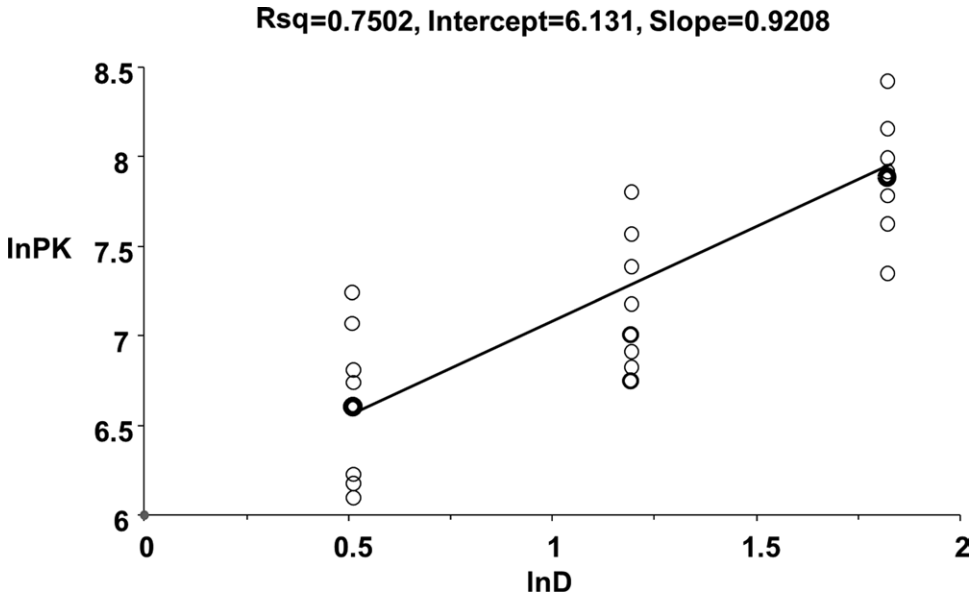

Figure 8. Diagram of relationship between single administration of hypoxanthine AUC_{0-t} and dose.

Conflicts of interest statement

The authors declare no conflict of interest.

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Author contributions

Yanchao Xing wrote and revised the article; Xianrui Wang established experimental methods; Xiaoming Wang conducted literature research; Xizi Zhao and Yaqing Guo drew pictures; Yuhong Huang processed data; Tekleab Teka modified the language of the article; Guixiang Pan and Lifeng Han formulated overall research goals.

Ethical approval of studies and informed consent

The study followed the guidelines of the ethics committee of the Experimental Animal Center of Tianjin University of Traditional Chinese Medicine (ethical registration number: 20160712-02).

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