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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Content

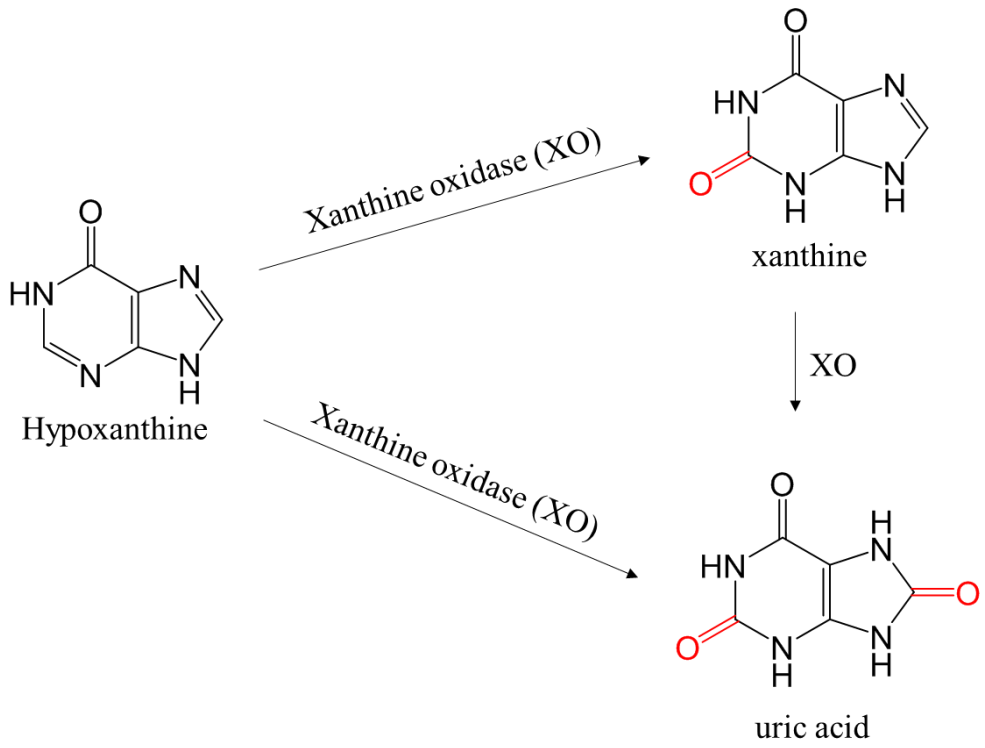

Figure S1. Transformation pathway of hypoxanthine

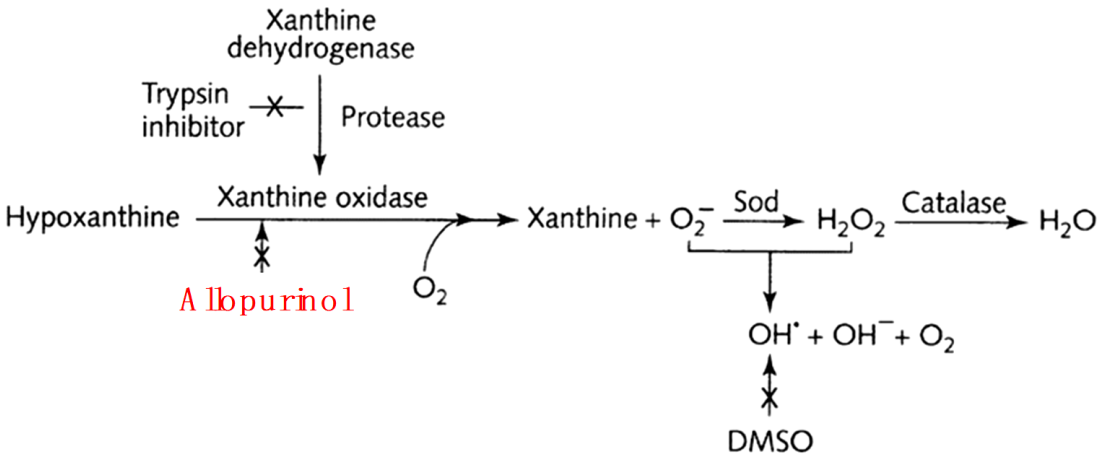

Figure S2. Allopurinol inhibits the metabolic pathway of hypoxanthine

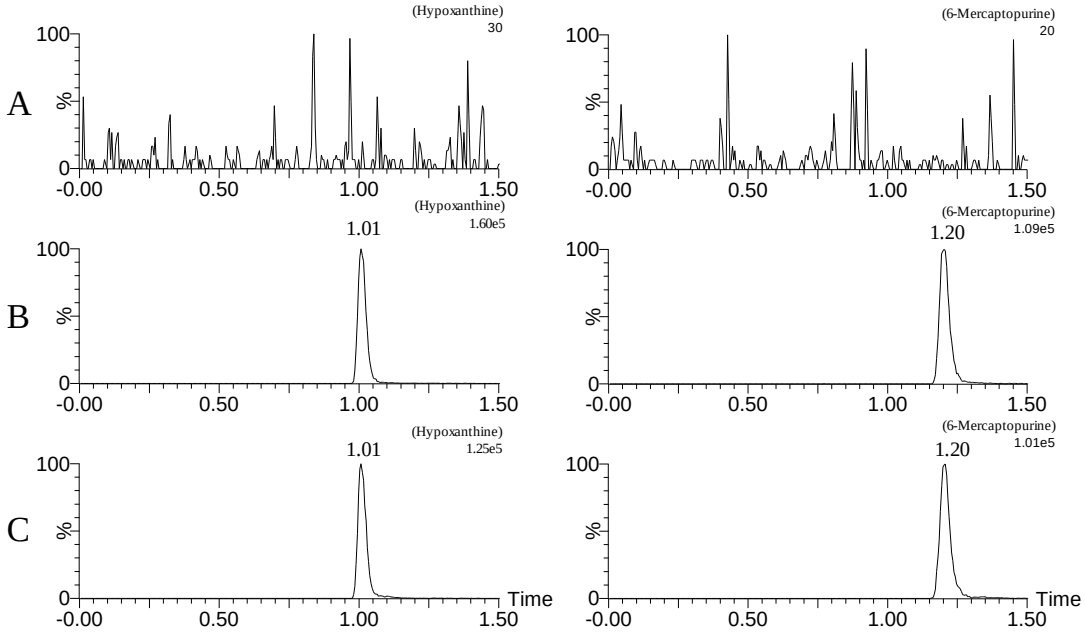

Figure S3. MRM chromatogram of hypoxanthine (target compound) and 6-mercaptopurine (IS1): A. The MRM chromatogram of blank saline B. The MRM chromatogram of Standard solution with internal standard C. The MRM chromatogram of sample solution with internal standard

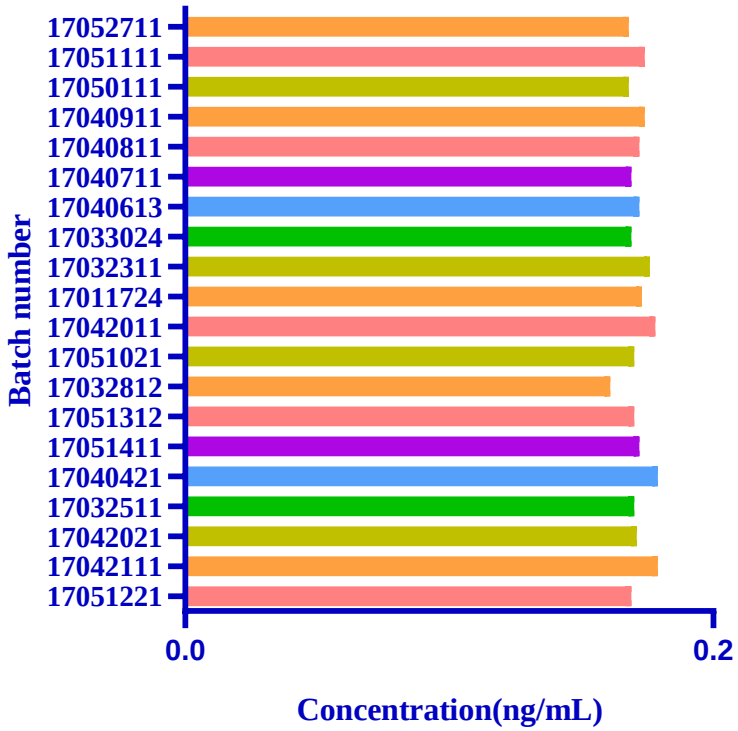

Figure S4. Histogram of the determination results of hypoxanthine in SXT injection of 20 batches using conventional LC/MS/MS without the introduction of standard addition method

Table S1. Precision and stability of hypoxanthine in SXT injection by using conventional LC/MS/MS without the introduction of standard addition method

Compound	Concentration (ng/mL)	Precision (RSD, %)		Stability	
		Intra-day	Inter-day	Average (%)	RSD (%)
Hypoxanthine	100	1.95	3.87	0.29	2.03
	300	1.88	5.36	0.69	2.33
	500	1.09	3.43	0.97	2.06

Table S2. Recovery of hypoxanthine in SXT injection by using conventional LC/MS/MS without the introduction of standard addition method

Serial number	Measured (μg/mL)	Addition (μg/mL)	Recovery (%)
1	109.8	25.00	100.20
2	108.4	25.00	94.60
3	110.4	25.00	102.60
4	109.2	25.00	97.80
5	109.7	25.00	99.80
6	109.8	25.00	100.20
7	148.5	62.50	102.00
8	145.7	62.50	97.52
9	147.0	62.50	99.60
10	148.0	62.50	101.20
11	146.5	62.50	98.80
12	144.8	62.50	96.08
13	171.8	87.50	99.49
14	169.3	87.50	96.63
15	174.6	87.50	102.69
16	170.3	87.50	97.77
17	172.7	87.50	100.51
18	171.4	87.50	99.03

Table S3. Repeatability of hypoxanthine in SXT injection by using conventional LC/MS/MS without the introduction of standard addition method

1	2	3	4	5	6	Average (mg/mL)	RSD (%)
337	339	340	339	336	343	339	0.71

Determination method of hypoxanthine in SXT injection without the introduction of the standard addition method

UPLC method

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 C18 (100mm×2.1mm, 1.7μm) maintained at 40 □. The mobile phase was 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, 85 % B-85 % B; 0.5-0.6 min, 15 % A-95 % A, 85 % B-5 % B; 0.6-1.0 min, 95 % A-95 % A, 5 % B-5 % B; 1.0-1.0 min, 95 % A-5 % A, 5 % B-95 % B; 1.1-3.5 min, 5 % A-5 % A, 95 % B-95 % B. The flow rate was 0.3 mL/min and the injection volume was 2 μL. Sample chamber temperature was 10 □.

Mass spectrometry method

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 multiple reactions monitoring (MRM) 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.

2.5 Method validation

2.5.1 Determination of hypoxanthine in SXT injection

The method of specificity was as follows. Physiological saline, sample solution, and sample solution spiked with hypoxanthine standard were injected and measured separately. Observe the chromatographic peak shape and resolution of hypoxanthine (target compound) and 6-Mercaptopurine (IS1) from impurities.

The method of precision was as follows. A mixed solution of low (100 ng/mL), medium (300 ng/mL), and high (500 ng/mL) containing 250 ng/mL internal standard with SXT injection of batch number 17040811 according to the "2.3.2" were prepared. These samples were measured six times a day as intra-day precision. The same sample of each concentration was detected once a day on the ground, and the next day and the third day as inter-day precision. Precision is the variation of the measured concentration, which is expressed in RSD.

The method of establishment of spiking curve was as follows. A mixed solution of 0, 100, 200, 300, 400 and 500 μL were prepared containing 250 ng/ml internal standard with SXT injection of batch number 17040811 according to the "2.3.2". The concentration (X) of the spiked hypoxanthine is used as the abscissa, and the peak area ratio (Y) of the target analyte to the internal standard is used as the ordinate and then weighting, correlation coefficient and so on were calculated.

The method of stability was as follows. 6 mixed solutions of low (100 ng/mL), medium (300 ng/mL), and high (500 ng/mL) were prepared containing internal standard of 250 ng/ml with SXT injection of batch number 17040811 according to the "2.3.2". These samples were detected at 0, 2, 4, 8, 12, 24 and 48 h. The area of hypoxanthine and internal standard peak were recorded, and the ratio of peak area and RSD were calculated.

The method of recovery was as follows. 6 mixed solution of low (100 ng/mL), medium (300 ng/mL), and high (500 ng/mL) containing internal standard of 250 ng/mL with SXT injection of batch number 17040811 were prepared according to the "2.3.2". The recovery rate is the percentage of the measured content plus a scalar.

The method of repeatability was as follows. SXT injection with batch number 17040811 was taken and 6 test solutions were prepared according to “2.3.2”.

2.5.2 Determination of hypoxanthine in plasma samples

The method of specificity was as follows. The blank plasma matrix without allopurinol, blank plasma matrix with allopurinol, plasma plus control substance and blood samples after administration were measured respectively. The specificity was judged by observing the separation of hypoxanthine (target compound) and 6-chloropurine (IS2) from impurities.

The methods of precision and accuracy were as follows. Quality control samples were prepared containing plasma matrix according to method "2.3.4", and the concentration was still 20 ng/mL、200 ng/mL and 3750 ng/mL. These three control concentrations were prepared for testing. Hypoxanthine peak area and internal standard peak area were substituted into the accompanying standard curve to calculate the concentration. Precision is the variation of the measured concentration, which is expressed in RSD. Accuracy is expressed as a percentage of the measured concentration to the true concentration. Intra-batch precision, inter-batch precision and accuracy were calculated separately after processing and measuring three batches of plasma samples.

The methods of linearity and range were as follows. Reference solutions containing plasma matrix were prepared according to method "2.3.4" and the concentrations were 10, 20, 50, 100, 200, 500, 1000 and 5000 ng/mL. The ratio of hypoxanthine to internal standard peak area (Y) was taken as the ordinate, while the hypoxanthine concentration (X) was taken as the abscissa. Weights, correlation coefficients and so on were then calculated.

The methods of matrix effects and extraction recovery were as follows. Extraction recovery and matrix effect were determined by measuring the quality control samples at low, medium, and high concentrations in six replicates. The extraction recovery was evaluated by comparing the analyte concentration in the plasma sample treated as described under "2.3.4" to the ratio of the analyte concentration determined by adding the standard after precipitation of the protein. The matrix effect was analyzed by

comparing the peak area of the analyte after addition of the precipitated protein of the standard with the peak area of the analyte in the unextracted sample.

The method of stability was as follows. Quality control samples containing plasma matrix were prepared according to method "2.3.4", and the concentrations were 20 ng/mL and 3750 ng/mL. The stability of hypoxanthine in quality control samples after 12 h and 24 h was investigated at room temperature. Short-term stability of hypoxanthine in quality control samples was performed within a 24 h at two concentration levels while long-term stability was performed at -70□ in a refrigerator for 15 days after three freeze-thaw cycles.

Table S4. Precision and stability of hypoxanthine in SXT injection

Compound	Concentration	Precision (RSD, %)		Stability	
	(ng/mL)	Intra-day	Inter-day	Average (%)	RSD (%)
Hypoxanthine	100	0.65	0.37	1.86	0.92
	300	0.82	0.85	2.84	0.51
	500	0.62	0.94	3.82	0.76

Table S5. Recovery of hypoxanthine in SXT injection

Serial number	Measured (μg/mL)	Addition (μg/mL)	Recovery (%)
1	99.26	100	99.26
2	98.29	100	98.29
3	96.70	100	96.70
4	97.18	100	97.17
5	101.44	100	101.44
6	99.39	100	99.39
7	301.90	300	100.63
8	300.78	300	100.26
9	298.84	300	99.61
10	291.90	300	97.30
11	291.47	300	97.16
12	302.85	300	100.95
13	498.77	500	99.75
14	495.31	500	99.06
15	505.15	500	101.03
16	492.61	500	98.52
17	503.52	500	100.70
18	493.70	500	98.74

Table S6. Repeatability of hypoxanthine in SXT injection

1	2	3	4	5	6	Average (mg/ml)	RSD (%)
0.126	0.134	0.132	0.132	0.131	0.126	0.130	2.4

Table S7. Precision and accuracy of hypoxanthine in plasma samples

Compound	Concentration (ng/mL)	Intra-batch		Inter-batch	
		Precision (RSD, %)	Acuracy (%)	Precision (RSD, %)	Acurac y (%)
Hypoxanthine	20.00	5.55	98.08	1.41	98.96
	200.00	1.47	85.33	0.58	85.85
	3,750.00	2.74	108.03	0.23	108.03

Table S8. Extract recovery and matrix effect of hypoxanthine in plasma samples

Compound	Concentration (ng/mL)	Extract recovery		Matrix effect	
		Precision (RSD, %)	Average (%)	Precision (RSD, %)	Average (%)
Hypoxanthine	20.00	3.81	79.49	1.72	101.48
	200.00	2.17	87.65	1.37	100.95
	3,750.00	2.47	101.63	1.08	101.14

Table S9. Stability of hypoxanthine in plasma samples

Processing condition	20.00 ng/mL		3750.00 ng/mL	
	Average (%)	(RSD, %)	Average (%)	(RSD, %)
12h stability in 10□	112.53	0.90	112.55	1.74
Room temperature 24h stability	113.38	1.49	113.49	0.97
Three freeze-thaw cycles stability	107.95	3.21	113.17	3.09
Short-term stability	113.18	0.79	112.58	1.31
Long-term stability	112.70	0.43	110.68	1.26