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Short communication

Quantification of andrographolide sodium bisulphite in urine after intravenous injection to rats by LC–MS/MS

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ABSTRACT

A liquid chromatography–tandem mass spectrometry method for the determination of andrographolide sodium bisulphite (ASB) in rat urine was established and validated. To our knowledge, the analytical method is the first developed assay for the determination of ASB in urine samples. Dehydroandrographolide (DAG) was used as an internal standard. ASB and DAG were separated on a C_{18} column and detected at negative ion mode using the mass transitions of m/z 413.2 → 287.2 and m/z 331.2 → 303.3, respectively. Good linearity was obtained over the range of 50–5000 ng/mL and the correlation coefficient was better than 0.99. The intra- and inter-day accuracy at all levels fell in the ranges of 85.8–101.4% and 87.9–97.5%, and the intra- and inter-day precision (RSD) were in the ranges of 4.3–11.2% and 8.4–13.3%, respectively. The recovery ranged from 96.1% to 98.3% and the matrix effects from 96.2% to 98.1%. Good stability was found under tested conditions. The method was successfully applied to a urinary excretion study of ASB in rats following intravenous administration of 80 mg/kg ASB.

1. Introduction

Andrographolide sodium bisulphite (ASB) is a water-soluble derivative of insoluble andrographolide, a major diterpenoid lactone found in the important traditional Chinese medicine, *Andrographis paniculata* Nees. ASB has been widely used as an injection formulation (Lianbizhi®) for the treatment of upper respiratory tract infection, bacillary dysentery, pneumonia and acute tonsillitis in China for several decades [1]. Between 1988 and 2005, however, 50 cases of adverse reactions were reported by State Food and Drug Administration of China, including acute renal damage, rash, dizziness, anaphylactoid reaction, anaphylactic shock and acute renal failure, of which 17 cases were acute renal failure [2]. It is necessary to evaluate the safety, pharmacokinetics and metabolism of ASB in preclinical experiments and clinical trials, especially the urinary excretion. Unfortunately, no pharmacokinetic or disposition investigation of ASB in animal or human is available.

In the present investigation, we have developed a liquid chromatography-tandem mass spectrometry (LC–MS/MS) method, with an electro-spray ionization source (ESI) operated at negative ion mode, for the quantification of ASB in rat urine. To our knowledge, it is the first published method for the determination of ASB in biological matrix although there are some studies for the determination of andrographolide in biological matrix [3–5]. After having been fully validated, it is applied to a urinary excretion study with a single dose of 80 mg/kg ASB intravenously injected to rats.

2. Materials and methods

2.1. Materials

ASB and an internal standard (IS) dehydroandrographolide (DAG) were provided by National Institutes for Food and Drug Control (Beijing, China). HPLC grade methanol was purchased from Fisher (Fair Lawn, NJ, USA). Water used for the LC–MS/MS was prepared using PWU-400 water purification system from Advantec (Tokyo, Japan). Saline for injection was obtained from Tianjin Pharmaceuticals (Jiaozuo, China). Blank rat urine was collected from normal rats individually housed in metabolic cages and stored at −70 °C.

2.2. Instrumentation and experimental conditions

A Thermo Fisher TSQ LC–MS/MS system consisted of an Accela Autosampler, an Accela pump and a Quantum Access triple quadrupole mass spectrometer. Data acquisition was performed with the Xcalibur software version 2.0, and data processing was carried out using the Thermo LCquan 2.5.6 data analysis program.

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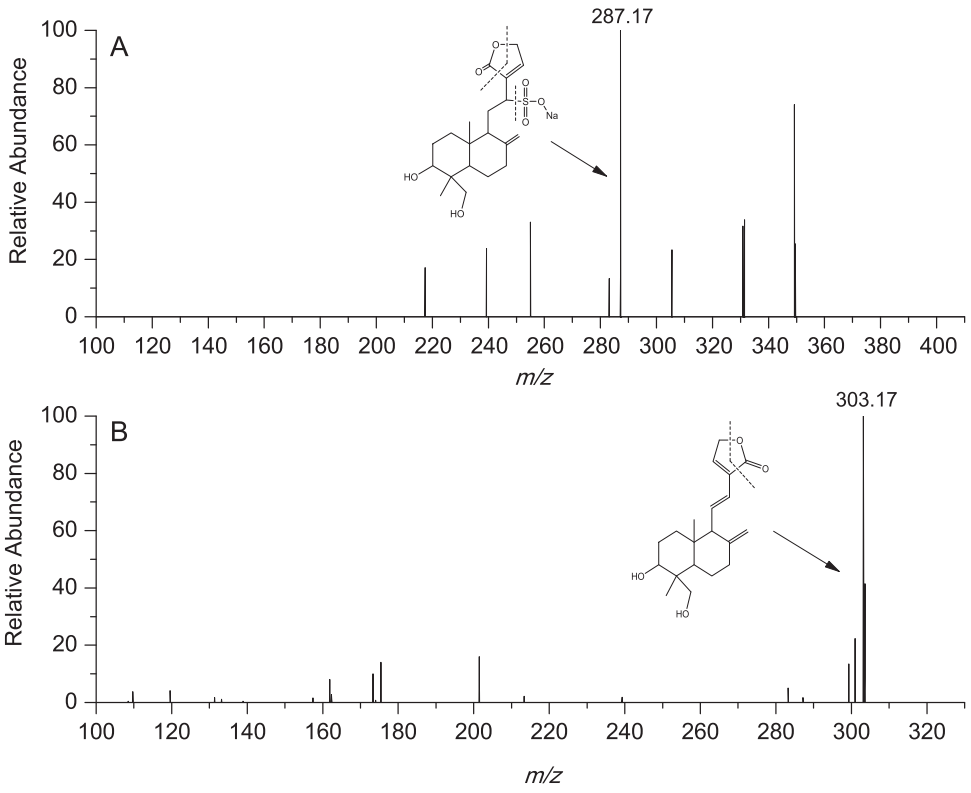

Fig. 1. Mass spectra of product ions of (A) ASB and (B) dehydroandrographolide in negative electrospray ionization mode.

Chromatographic column used was a Hypersil Gold C_{18} (50 × 2.1 mm, 1.9 μm particle size, Thermo Fisher Inc., Waltham, MA, USA) equipped with a guard cartridge of Phenomenex C_{18} (4 × 3.0 mm, Shimadzu Inc., Tokyo, Japan) and was maintained at 35 °C. Separation was performed using a mobile phase of 20:80 (*v*/*v*) methanol:water held isocratically for 0.5 min, followed by a 0.5 min linear gradient to 80:20 (methanol:water), and then by a 4 min isocratic elution before to return to the initial conditions in 0.1 min at a flow rate of 0.2 mL/min. After 0.9 min of re-equilibration, the column was ready for a next injection. The autosampler tray was maintained at ambient temperature, the injection volume was 10 μL and the injection port was washed between runs with 2 mL of 50% methanol aqueous solution. Quantification was done using selected reaction monitoring (SRM) mode to monitor precursor-product ion transitions of *m*/*z* 413.2 → 287.2 for ASB, 331.2 → 303.3 for the IS at negative ionization mode (Fig. 1). The optimal instrument conditions were as follows: capillary temperature of 300 °C, spray voltage of 4000 V, sheath gas of 15, auxiliary gas of 5. Nitrogen was used as both sheath gas and auxiliary gas, and argon was employed as a collision gas at a pressure of 1.5 m Torr. The collision energy was 30 and 26 eV for ASB and the IS, respectively. Scan time was 0.2 s per transition.

2.3. Sample preparation

ASB was weighed out in duplicate and dissolved in water to yield two individual stock solutions designated stock and stock quality control (QC) solutions at levels of 1 mg/mL, respectively. A series of ASB working solutions at a range of 0.5–50 μg/mL were obtained by further diluting stock solution. IS stock solution of 1 mg/mL was prepared by dissolving requisite amount in methanol and IS working solution of 10 μg/mL was obtained by diluting the stock of 1 mg/mL in methanol.

The calibration standards and QC samples (LLOQ, lower limit of quantification; LQC, low QC; MQC, middle QC; HQC, high QC; ULOQ, upper limit of quantification) were prepared by spiking 90 μL of blank urine with 10 μL of working solutions. Calibration standards were made at 50, 100, 200, 500, 800, 1000, 2000 and 5000 ng/mL. QC samples were prepared at 50 ng/mL (LLOQ), 100 ng/mL (LQC), 500 ng/mL (MQC), 2000 ng/mL (HQC) and 5000 ng/mL (ULOQ). The spiked urine samples at all the levels were stored at −70 °C for validation and sample analysis.

The 100 μL of urine sample and 10 μL of IS solution were added into a 1.5-mL polypropylene centrifuge micro-tube. Following the addition of 100 μL of methanol, the sample in the capped tube was vigorously vortex-mixed for 1 min and then centrifuged at 13,400 × *g* for 5 min. The supernatant was transferred to a clean vial and a 10-μL aliquot of the sample was injected into LC–MS/MS. Those samples with concentrations above ULOQ were diluted with blank urine and reanalyzed.

2.4. Method validation

The method was validated for selectivity, linearity, accuracy, precision, recovery and stability according to the FDA guidelines for the validation of bioanalytical methods [6].

2.4.1. Selectivity

The selectivity of the method against endogenous interferences was verified by the analysis of blank and spiked LLOQ samples prepared using six different sources of non-pooled, analyte-free urine to determine the extent to which endogenous urine components might contribute to the interference at the retention time of ASB and the IS. If interference exits, the signal should not exceed 20% of LLOQ peak area and 5% of IS peak area.

2.4.2. Linearity

The linearity of the method was evaluated by five calibration curves in a range of 50–5000 ng/ml of ASB, including LLOQ. The calibration curve was constructed by plotting the peak area

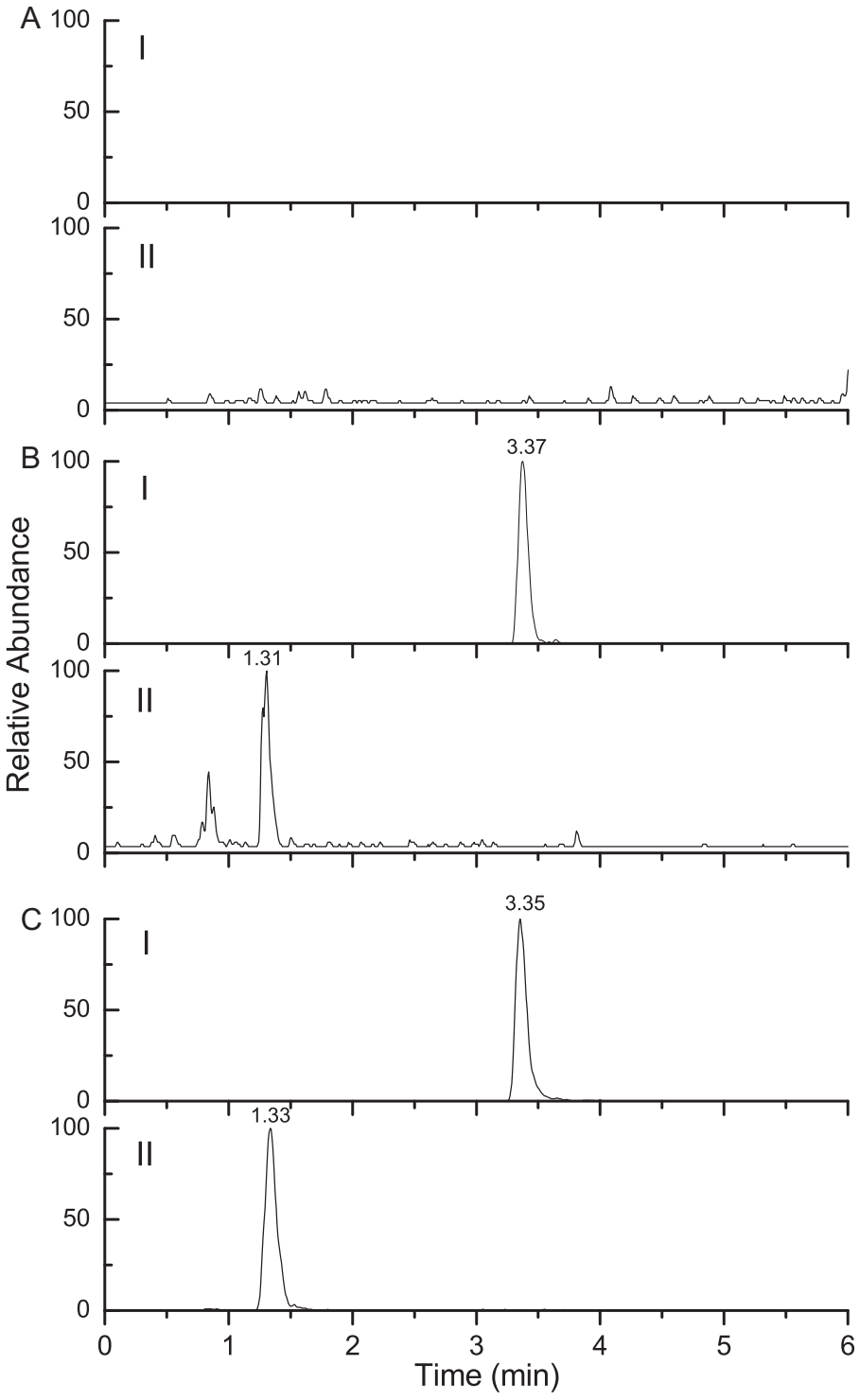

Fig. 2. Representative SRM chromatograms (I: *m*/*z* 331.2 → 303.3 for dehydroandrographolide; II: *m*/*z* 413.2 → 287.2 for ASB) for (A) blank urine; (B) urine spiked with ASB (50 ng/mL) and dehydroandrographolide; (C) urine sample collected at an interval of 24–48 h after intravenous administration of 80 mg/kg ASB.

ratios of ASB to the IS against the concentration of ASB using least-squares linear regression analysis. The acceptance criterion for each back-calculated standard concentration should be within 15% of the nominal value, except it should not exceed 20% at the LLOQ.

2.4.3. Accuracy and precision

Intra- and inter-day (on three consecutive days) accuracy and precision of the assay were evaluated at four different concentrations (LLOQ, LQC, MQC and HQC) in six replicates against calibration standards. The accuracy and precision were calculated and expressed as the percentage value of observed concentration to theoretical concentration and the relative standard deviation (RSD), respectively. For acceptable intra- and inter-day values, the accuracy should be within 85–115% with an exception of 80–120% for LLOQ level. The precision at each concentration level from the nominal concentration was expected to be within ±15% except LLOQ, for which it should be within ±20%.

Table 1
Accuracy, precision, matrix effect and recovery of the method. Data are expressed in percentage ($n = 6$).

Concentration (ng/mL)	Intra-day		Inter-day		Matrix effect	Recovery
	Accuracy	RSD	Accuracy	RSD		
50	85.8	11.2	87.9	13.3	–	–
100	91.9	10.6	93.6	11.4	96.2 ± 8.9	96.1 ± 9.7
500	98.2	4.3	96.7	8.4	97.4 ± 11.5	93.7 ± 8.5
2000	101.4	8.8	97.5	10.2	98.1 ± 6.1	98.3 ± 10.2

–, not performed.

2.4.4. Matrix effects and recovery

Matrix effects and recovery were performed at LQC, MQC and HQC levels ($n = 6$, for each concentration). The matrix effects were expressed as the ratios of the mean peak areas of the analyte spiked post-treatment to those of the mean peak areas of the neat standards at corresponding concentrations. The recovery was calculated by comparing the peak areas obtained from treated spiked samples with those of ASB neat solutions at corresponding concentrations. The recovery of the IS was also evaluated using the same procedure. The RSD value of matrix effects at each concentration should be less than 15%.

2.4.5. Dilution integrity

The dilution integrity experiment was performed with a purpose to validate the dilution test to be carried out on higher analyte concentrations (above ULOQ), which may be encountered during real rat samples analysis. Dilution integrity experiment was performed at 500 μg/mL (100-fold of upper limit of quantitation) and 200 μg/mL (100-fold of high quality control). Six replicate samples were diluted to 1/200 (2500 and 1000 ng/mL) by spiking blank rat urine and their concentrations were calculated by applying the dilution factor of 200 against calibration curve.

2.4.6. Stability

Stability experiments were performed to evaluate the analyte stability in urine samples under different conditions, simulating the same conditions which occurred during sample collection, handling, storage and analysis. Stability of ASB in urine was assessed at LQC, MQC and HQC levels using triplicate at each level for three freeze–thaw cycles, short-term temperature, post-preparative and long-term temperature stabilities. The freeze–thaw stability was evaluated over three freeze–thaw cycles within three days. In each freeze–thaw cycle, the spiked urine samples were frozen for 24 h at −70 °C and completely thawed at room temperature for 30 min. In short-term temperature stability, three aliquots of each concentration were thawed unassisted at ambient temperature and kept at this temperature for 4 h. The post-preparative stability of the extracted urine samples was determined after keeping the processed samples at ambient temperature for 6 h. The long-term stability was evaluated after keeping the urine samples frozen at −70 °C for 7 consecutive days. Thereafter, samples were analyzed and the resulted values for these samples were compared to those of the respective freshly prepared QC samples. ASB was considered stable in the different conditions when a deviation of less than ±15% from the actual value was obtained.

2.5. Excretion study

All procedures involving animals were approved by our Institutional Animal Care and Use Committee. Adult male and female Sprague-Dawley rats, weighing 243 ± 12 g, were obtained from Vital River Laboratories (Beijing, China) and individually housed in metabolic cages and allowed free movement and access to food and water during the whole experiment. The dosing solution of ASB (20 mg/mL) was dissolved in saline. A single 80 mg/kg dose of ASB was administered into the tail veins of rats that were under mild isoflurane anesthesia. Urine of rats following administration was collected during the intervals of 0–4, 4–10, 10–24, 24–48, 48–72 h. The exact volume of urine was measured and stored at −70 °C until analysis within a week. At the end of the experiments, animals were sacrificed using carbon dioxide.

3. Results and discussion

3.1. Method development

ASB and the IS could be ionized under either positive ESI (ESI^+) or negative ESI (ESI^-) modes, however signal intensity and stability were much better at ESI^- than ESI^+, therefore, the ESI^- mode was used for the analyte quantification. ASB and the IS gave predominant singly charged precursor $[M-Na]^-$ and $[M-H]^-$ ions at m/z of 413.2 and 331.2 respectively in Q1 MS full scan spectra. Mass spectrometric parameters were suitably optimized so as to achieve the maximum abundance of product ions of ASB and the IS. Fragmentation was initiated using sufficient argon for collision induced dissociation and by applying 20 eV collision energy to break the precursor ions. The most abundant ions found in the product ion mass spectra were m/z 287.2 and 303.3 at 30 and 26 eV collision energy for ASB and the IS, respectively (Fig. 1A and B).

The chromatographic conditions were aimed at getting adequate response and sharp peak shape for ASB and the IS. The applied chromatographic method ensured the elution of both compounds within 6.0 min and produced symmetric peaks. Different volume ratios of methanol–water and acetonitrile–water combinations were explored as a mobile phase. It was observed that gradient condition of methanol and water as the mobile phase was most appropriate for faster elution, better efficiency and peak shape. Isocratic mobile phase of methanol-water at any ratio produced broad, tailed and asymmetric peak of ASB.

An internal standard is necessary for the determination of analytes in biological samples, and DAG with a similar structure to ASB was found to be the optimal IS for our target compound. Sample pretreatment plays a key role in determination of drugs in biological matrix, and methanol rather than acetonitrile was selected due to its compatibility with the mobile phase to produce symmetric peak shapes for the analyte and the IS.

3.2. Selectivity

The aim of performing selectivity was to ensure the authenticity of the results for study sample analysis. Fig. 2 demonstrates the selectivity results with the chromatograms of blank urine and the peak response of ASB at LLOQ level. Also, the real rat urine sample chromatogram is presented for ASB at an interval of 24–48 h after intravenous administration of 80 mg/kg ASB. The chromatograms showed excellent peak shape for both the analyte and the IS. No endogenous interferences were found at the retention times of ASB (1.31 min) and DAG (3.37 min) in the blank plasma. The area observed at the retention time of ASB was much less than 20% of

its LLOQ area and it was greatly less than 5% IS area observed in the LLOQ sample.

3.3. Linearity, accuracy and precision

The calibration curves were linear from 50 to 5000 ng/mL with correlation coefficient $r \geq 0.9975$ across five regression curves. The equation for means of five calibration curves was $R = 0.000987C - 0.02796$, where R is the peak area ratios of ASB to the IS and C is ASB concentration. The standard deviation values obtained for slope and intercept from five curves was 0.000110 and 0.02143, respectively. The non-zero standards showed less than 20% deviation at 50 ng/mL and 1.6–8.9% deviation at all other concentrations. The peak area ratio values of calibration standards were proportional to the concentration of the drug in urine over the tested range. LLOQ of ASB was set at 50 ng/mL, at which the precision of 20% and accuracy of 80–120% were achieved from the data listed in Table 1. The calculated value of signal to noise (S/N) was approximately 700 at this concentration, indicating that the LLOQ could be made lower than 50 ng/mL if it was necessary. But this LLOQ (50 ng/mL) showed much enough sensitivity for measuring ASB in rat urine at the last sampling time point after intravenous injection at a single dose of 80 mg/kg.

The intra- and inter-day accuracy and precision were evaluated by analysis of LLOQ, LQC, MQC and HQC samples with six determinations per concentration on the same day over three days. The concentration of each sample was calculated from calibration curve. The results of the accuracy and precision are shown in Table 1. The intra- and inter-day accuracy for ASB at 50, 100, 500 and 2000 ng/mL levels in rat urine fell in the ranges of 85.8–101.4% and 87.9–97.5%, and the intra- and inter-day precision (RSD) were in the ranges of 4.3–11.2% and 8.4–13.3%, respectively. These results demonstrated that the values were within the acceptable range, and the method was sufficiently accurate and precise.

3.4. Matrix effects, recovery and dilution integrity

The results of matrix effects and recovery are summarized in Table 1. The matrix effects for ASB at concentrations of 100, 500 and 2000 ng/mL were all within 85–115% with RSD of not more than 15%. The matrix effects on the ionization of the analyte were not obvious under these conditions.

The overall mean recoveries for ASB at LQC, MQC and HQC levels were 96.1 ± 9.7%, 93.7 ± 8.5% and 98.3 ± 10.2%, respectively. The recovery of the IS was found to be 98.2% with a RSD value of 5.1%. The recovery of ASB and the IS in rat urine was consistent, precise and reproducible.

The mean back-calculated concentrations for 1/200 dilution samples at two concentrations were within 85–115% of their nominal values. The precision (RSD) for 1/200 dilution samples was ≤6.3% at both levels.

3.5. Stability

ASB was found stable in urine sample at room temperature for at least three freeze–thaw cycles and a minimum period of 4 h. The analyte in extracted urine samples was stable for 6 h under room temperature. The spiked urine samples of ASB stored at −70 °C for long-term stability were found stable for a minimum period of 7 days. The RSD values for ASB in all the stability studies were within 1.3–8.1%. The values for the percentage change for the above stability experiments are compiled in Table 2.

Table 2
Stability results for ASB in rat urine ($n = 3$).

Stability conditions	Level	Accuracy (%)	RSD (%)
Three freeze–thaw cycles (−70 °C)	LQC	98.3	6.4
	MQC	97.6	1.3
	HQC	98.1	4.7
Short-term (4 h, room temperature)	LQC	99.1	4.7
	MQC	97.6	7.1
	HQC	99.2	2.3
Post-preparative (6 h, room temperature)	LQC	98.5	3.9
	MQC	98.6	4.3
	HQC	100.1	3.3
Long-term (7 days, −70 °C)	LQC	98.8	4.9
	MQC	99.1	8.1
	HQC	100.6	5.7

3.6. Excretion study

The developed and validated method was applied to a urine excretion study of ASB in rats following intravenous administration of 80 mg/kg ASB. The method was sensitive enough to determine ASB urine concentration up to 72 h. Those samples with concentrations above the upper calibrator were diluted 1/200 with blank urine and reanalyzed. Following intravenous administration of ASB, a major proportion of ASB disposed renally was eliminated within 4 h (∼85% of total urinary excretion) and renal excretion was complete at 72 h. The cumulative amounts of ASB excreted in urine within 72 h as parent drug were 3262.7 ± 650.1 μg (16.92 ± 3.95% of administered dose).

4. Conclusion

A specific and sensitive method was fully validated for the determination of ASB in urine by LC–MS/MS. Very good accuracy, precision and recovery were obtained and no significant matrix effect was observed. The method was successfully applied to measurement of ASB in urine after a single dose of 80 mg/kg ASB was intravenously injected to rats.

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