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Short communication

Determination of andrographolide sodium bisulphite in Beagle dog plasma by LC–MS/MS and its application to pharmacokinetics

Shuang-Qing Zhang*, Yu-Ming Fan**

National Institutes for Food and Drug Control, A8 Hong Da Zhong Lu, Beijing Economic-Technological Development Area, Beijing 100176, China

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ABSTRACT

A sensitive and reliable liquid chromatography–tandem mass spectrometry (LC–MS/MS) has been developed and validated for the determination of andrographolide sodium bisulphite (ASB) in dog plasma using dehydroandrographolide (DAG) as an internal standard. Chromatographic separation was achieved on a Hypersil Gold C_{18} column (50 mm × 2.1 mm, 1.9 μm) with gradient elution that consisted of methanol and water at a flow rate of 0.2 mL/min. Quantification was done using selected reaction monitoring (SRM) mode to monitor precursor–product ion transitions of m/z 413.2→287.2 for ASB and 331.2→303.3 for DAG at negative ionization mode. Good linearity was obtained over the range of 10–1000 ng/mL and the correlation coefficient was better than 0.99. The intra- and inter-day accuracies ranged from 97.2% to 107.8% and precisions (RSD) were within 13.9%. ASB was found stable under three freeze–thaw cycles, short-term temperature, post-preparative and long-term temperature conditions. The method was successfully applied to a pharmacokinetic study of ASB intravenously administered to Beagle dogs.

1. Introduction

Andrographolide is a major diterpenoid constituent of the plant *Andrographis paniculata Nees* (Family Acanthaceae), the leaf extract of which is an important herbal medicine widely used in China, Japan, Korea, India and other Asia countries for more than 2000 years. The poor oral bioavailability of andrographolide associated with high lipophilicity (log $P = 2.632 \pm 0.135$) and low aqueous solubility (3.29 ± 0.73 μg/mL) is a major challenge in clinical efficacy [1]. To improve solubility and bioavailability, andrographolide sodium bisulphite (ASB, Fig. 1A), a water-soluble active analogue of andrographolide, has been synthesized and clinically used for the treatment of upper respiratory tract infection, bacillary dysentery, pneumonia and acute tonsillitis over the past 40 years [2]. Recently, adverse reactions including acute renal failure caused by ASB were reported, however the mechanism still remained unclear. It is very imperative to evaluate the safety and pharmacokinetics of ASB in animals. To our knowledge, the determination of ASB in dog plasma for pharmacokinetic evaluation of ASB has not been reported. Although our previous assay for the determination of ASB in rat urine was established [3], strong interference for the measurement of ASB in dog plasma was found using the previous method, therefore some changes should be made to remove the interference, such as MS parameters and mobile phase. Five-time lower LLOQ value of 10 ng/mL was obtained employing the present method, compared to 50 ng/mL in the previous method. Additionally, the biological matrix was dog plasma in the present study instead of rat urine in our former method, and full validation should be carried out according to the EMA guideline [4]. The purpose of the present study was to quantify plasma levels of ASB in Beagle dog after intravenous administration of ASB.

2. Materials and methods

2.1. Materials

ASB and an internal standard (IS) dehydroandrographolide (DAG, Fig. 1B) were supplied by National Institutes for Food and Drug Control (Beijing, China). HPLC grade methanol was purchased from Fisher (Fair Lawn, NJ, USA). All other reagents were analytical grade and obtained through commercial sources.

2.2. Instrumentation and experimental conditions

The biological sample analysis was performed on a Thermo Fisher TSQ LC–MS/MS system equipped with an Accela autosampler, an Accela pump and a Quantum Access MAX triple quadrupole mass spectrometer. Data acquisition was performed with the Xcalibur software, and data processing was carried out using the Thermo LCquan data analysis program. Separation was achieved

* Corresponding author. Tel.: +86 10 67872233x8220; fax: +86 10 67876255.
** Corresponding author. Tel.: +86 10 67095395; fax: +86 10 67033529.
E-mail addresses: zhangshq@hotmail.com (S.-Q. Zhang), fym60@yahoo.com.cn (Y.-M. Fan).

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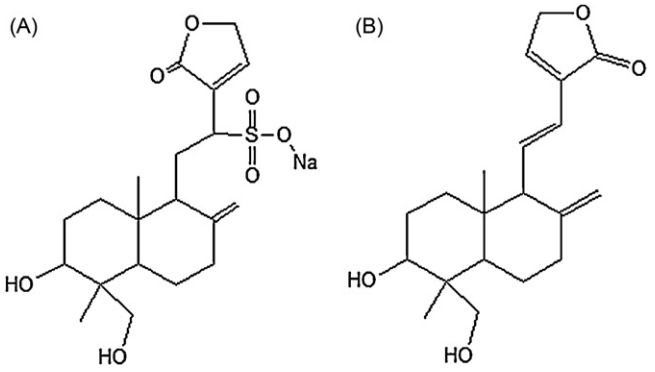

Fig. 1. Chemical structure of ASB (A) and dehydroandrographolide (B).

on a Hypersil Gold C_{18} column (50 mm × 2.1 mm, 1.9 μm particle size, Thermo Fisher Inc., USA) using a mobile phase that consisted of methanol (A) and water (B) with the following gradient program (v/v): 10% A at 0–0.8 min, 10% A → 90% A at 0.8–1.0 min, 90% A at 1.0–5.0 min, 90% A → 10% A at 5.0–5.1 min, and 10% A at 5.1–6.0 min. The flow rate was 0.2 mL/min, the injection volume was 10 μL and the column oven temperature was set at 35 °C.

Quantification was done using selected reaction monitoring (SRM) mode to monitor precursor–product ion transitions of *m/z* 413.2→287.2 for ASB and 331.2→303.3 for the IS at negative ionization mode. The optimal instrument conditions were as follows: capillary temperature of 300 °C, spray voltage of 3500 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

Individual standard stock solutions of ASB (1 mg/mL) and DAG (1 mg/mL) were prepared by accurately weighing the required amounts into separate volumetric flasks and dissolving in methanol. IS working solution of 10 μg/mL was obtained by diluting the stock of 1 mg/mL in methanol.

The calibration standard for ASB was prepared by spiking 4.95 mL of blank dog plasma with 50 μL of ASB stock solution. The resulting plasma standard had a concentration of 10 μg/mL. Further dilutions were made from this stock with blank plasma to afford plasma standards in the range of 10–1000 ng/mL. Quality control (QC) samples were prepared at 10 ng/mL (LLOQ, lower limit of quantification), 30 ng/mL (LQC, low QC), 400 ng/mL (MQC, middle QC), 750 ng/mL (HQC, high QC) and 1000 ng/mL (ULOQ, upper limit of quantification). The spiked plasma samples at all the levels were stored at −70 °C for validation and sample analysis.

The 100 μL of plasma sample and 10 μL of IS working solution were added into a 1.5-mL polypropylene centrifuge micro-tube. Following the addition of 200 μL of methanol, the sample in the capped tube was vigorously vortex-mixed for 5 min and then centrifuged at 13,400 × *g* for 10 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 plasma and reanalyzed.

2.4. Method validation

The method validation of ASB in dog plasma was validated for selectivity, linearity, accuracy, precision, matrix effect, recovery, carry-over, dilution integrity and stability in accordance with the "Guideline on bioanalytical method validation" of the European Medicines Agency [4]. Test for selectivity was carried out in six different sources of non-pooled and analyte-free plasma, processed by the same protein precipitation protocol and analyzed to determine the extent to which endogenous plasma components may contribute to the interference at the retention time of the analyte and IS. Absence of interfering components is accepted where the response is less than 20% of LLOQ for the analyte and 5% for the IS.

The linearity of the method was evaluated by analysis of standard plots associated with an eight-point standard calibration curve in the range of 10–1000 ng/mL, including LLOQ. Five linearity curves containing eight non-zero concentrations were analyzed. Best-fit calibration curves of peak area ratio of ASB to the IS against concentration were constructed. The concentration of the analyte was calculated from calibration curve ($y = ax + b$, where y is the peak area ratio) using the least-squares linear regression analysis for ASB. 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. The regression equation for the calibration curve was also used to back-calculate the measured concentration at each QC level. 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. 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%.

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 the analyte neat solutions at corresponding concentrations. The Matrix effects and recovery of the IS were also evaluated using the same procedure. The RSD value of matrix effects at each concentration should be less than 15%.

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 dog samples analysis. Dilution integrity experiment was performed at 100 μg/mL (100-fold of ULOQ). Six replicate samples were diluted to 1/200 (500 ng/mL) by spiking blank dog plasma and their concentrations were calculated by applying the dilution factor of 200 against calibration curve. Accuracy and precision should be within ±15%.

Carry-over experiment was performed to verify any interference due to carry over of analytes, which may reflect in subsequent runs. Carry over was evaluated by the analysis of blank plasma samples spiked with the IS after the analysis of ULOQ (1000 ng/mL, $n = 3$) and after the analysis of highly concentrated samples (10,000 ng/mL, $n = 3$). Carry over in the blank sample following the high concentration standard should not be greater than 20% of LLOQ and 5% for the IS.

Stability of ASB in plasma was assessed at LQC 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 3 days. In each freeze–thaw cycle, the spiked plasma 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

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

Concentration (ng/mL)	Intra-day		Inter-day		Matrix effect	Recovery
	Accuracy	RSD	Accuracy	RSD		
10	105.3	14.3	107.8	13.9	/	/
30	97.2	11.6	102.5	12.3	94.9 ± 9.7	76.3 ± 8.4
400	102.1	4.9	99.6	5.3	95.9 ± 5.9	86.7 ± 4.5
750	99.3	7.1	103.1	6.7	97.1 ± 6.3	89.1 ± 5.6

/, not performed.

thawed unassisted at ambient temperature and kept at this temperature for 4 h. The post-preparative stability of the extracted plasma samples was determined after keeping the processed samples at ambient temperature for 6 h. The long-term stability was evaluated after keeping the plasma samples frozen at −70 °C for 10 consecutive days. Thereafter, samples were analyzed, the resulting values for these samples were compared to nominal concentration and expressed as a percentage of the nominal concentration. ASB was considered stable in the different conditions when a deviation of less than ±15% from the actual value was obtained.

2.5. Pharmacokinetic study

All studies on animals were in line with the guidelines for the Care and Use of Laboratory Animals. Three adult male Beagle dogs, weighing 9.4 ± 0.4 kg, were obtained from Marshall Farms (Beijing, China) and individually housed on a 12 h light-dark cycle. All dogs were kept under conditions of 25 °C and 50% relative humidity and were allowed free access to food and water. The dosing solution of ASB dissolved in saline was intravenously administered to dogs at a dose level of 50 mg/kg. At 0.083, 0.25, 0.5, 1, 2, 4 and 6 h, approximately 0.3 mL of blood samples was taken and centrifuged in heparin-coated microcentrifuge tubes to obtain plasma. Plasma sample was transferred to another microcentrifuge tubes. All the samples were stored at −70 °C until analysis within 10 days.

3. Results and discussion

3.1. Method development

As the literature reveals, there is no report yet on the determination of ASB in plasma by LC–MS/MS. Thus, in the present investigation method development was initiated to realize a rugged, sensitive, and selective LC–MS/MS method for the quantification of ASB in dog plasma. To accomplish this purpose it was imperative to have a simple, inexpensive and an efficient treatment procedure, with a short chromatographic run time. Also, the sensitivity should be adequate enough to monitor at least three to five half-lives of ASB concentration with good accuracy and precision for dog plasma samples.

The tuning of MS parameters was carried out in positive as well as negative ionization modes for ASB and the IS using 200 ng/mL tuning solution. The response observed was much better in the negative ionization mode for both compounds compared to the positive mode. In Q1 MS full scan spectra, the analytes gave predominant singly charged precursor $[M-Na]^-$ ions at m/z of 413.2 for ASB and precursor $[M-H]^-$ ion at m/z of 331.2 for IS, respectively. The most abundant ions found in the product ion mass spectra were m/z 287.2 and 303.2 at 30 and 26 eV collision energy for ASB and the IS, respectively. Methanol not liquid–liquid extraction solvents was used for the extraction of ASB in dog plasma due to its water-soluble property.

3.2. Selectivity

The aim of selectivity was to ensure the authenticity of the results in sample analysis. Fig. 2 shows typical chromatograms of blank plasma and spiked plasma at LLOQ level of ASB. No significant direct interference in the blank plasma was observed from endogenous substances in drug-free dog plasma at the retention time of both compounds (2.72 min) or the IS (3.53 min). 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 at the LLOQ level.

3.3. Linearity, accuracy and precision

The calibration curve equation was $y = (0.000474 \pm 0.000018)x \times (0.023732 \pm 0.001535)$ with correlation coefficient of 0.9965 ± 0.0031. The non-zero standards showed less than 20% deviation at 10 ng/mL and 4.6–11.2% deviation at all other concentrations. The peak area ratio values of calibration standards were proportional to the concentration of the drug in plasma over the tested range. LLOQ value of ASB was set at 10 ng/mL, at which the precision of 20% and accuracy of 80–120% were achieved from the data listed in Table 1.

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 3 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 of ASB at low-to-high concentrations ranged from 97.2% to 107.8%, and precision was within 14.3%. These data indicated that the LC–MS/MS method was reliable and reproducible.

3.4. Matrix effects, recovery, dilution integrity and carry-over

The results of matrix effects and recovery are presented in Table 1. RSD values of matrix effects at concentrations of 30, 400 and 750 ng/mL were in the range of 5.9–9.7%. 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 76.3%, 86.7% and 89.1% with RSD of less than 8.4%. Thus, the consistency in recoveries of ASB supported the treatment procedure for its application to routine sample analysis. The matrix effects and recovery of the IS were 71.2 ± 3.1% and 83.4 ± 3.9%.

The dilution integrity test, the dilution 1/200 of high concentrated samples (100 μg/mL), demonstrated an accuracy of 95.1% and an RSD of 8.3% for the diluted plasma samples. Moreover, no carryover was observed in the analysis of a blank plasma sample injected after the analysis of the upper calibrator (1000 ng/mL), or after a highly concentrated sample (10,000 ng/mL).

3.5. Stability

ASB was found stable in plasma sample for at least three freeze–thaw cycles and a minimum period of 4 h at room

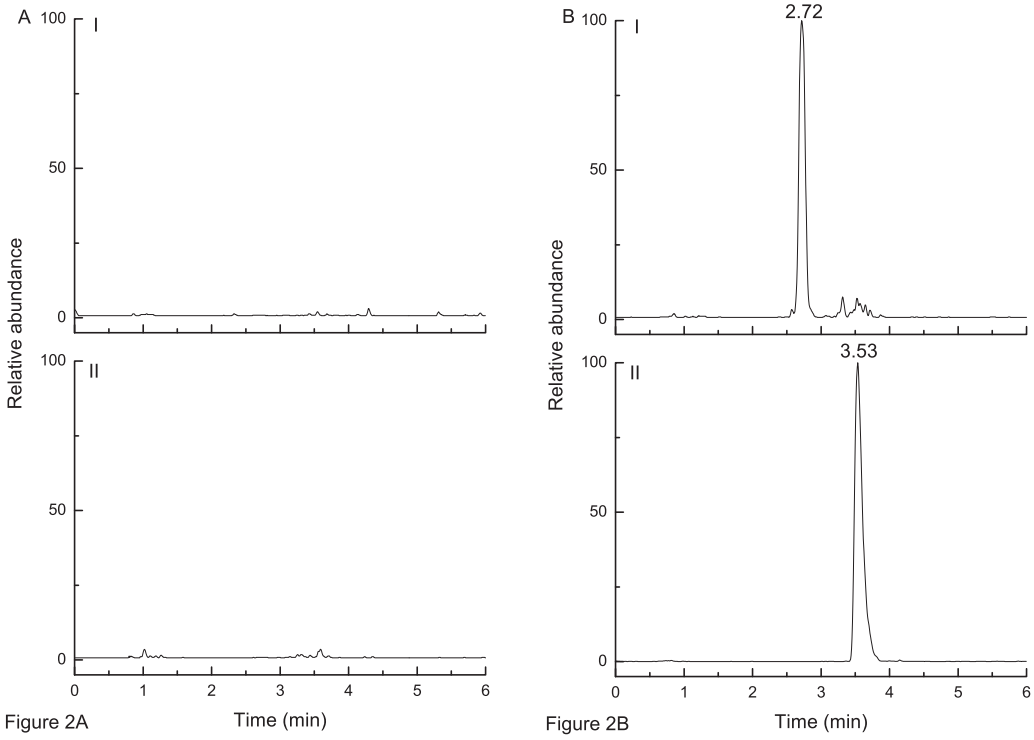

Figure 2A Figure 2B Figure 2C

Fig. 2. Representative SRM chromatograms (I: m/z 413.2→287.2 for ASB; II: m/z 331.2→303.3 for dehydroandrographolide) for (A) blank plasma; (B) a plasma sample spiked with ASB at LLOQ level; (C) a plasma sample obtained 5 min after intravenous administration of ASB at a dose of 50 mg/kg.

temperature. The analyte in processed plasma samples was stable for 6 h under room temperature. The spiked plasma samples of ASB stored at −70 °C for long-term stability were found stable for a minimum period of 10 days. The accuracy values for ASB at the levels of 30 and 750 ng/mL in all the stability studies fell in the range of 96.4–102.7% and the RSD were within 4.2–8.9%. The values for the percentage change for the above stability experiments are compiled in Table 2.

3.6. Pharmacokinetic study

The developed and validated LC–MS/MS method was applied to a pharmacokinetic study of ASB in dogs following intravenous administration. The typical chromatogram of incurred sample from dosed dogs is presented in Fig. 2C. The concentration–time profile of ASB in plasma is shown in Fig. 3. Non-compartmental analysis was employed to calculate pharmacokinetic parameters.

Table 2
Stability results for ASB in dog plasma ($n = 3$).

Stability conditions	Level	Accuracy (%)	RSD (%)
Three freeze–thaw cycles (−70 °C)	LQC	96.4	8.5
	HQC	97.8	6.2
Short-term (4 h, plasma at room temperature)	LQC	98.6	8.9
	HQC	98.5	4.4
Post-preparative (6 h, extract at room temperature)	LQC	97.2	5.7
	HQC	102.7	4.2
Long-term (10 days, −70 °C)	LQC	97.1	8.4
	HQC	98.4	6.3

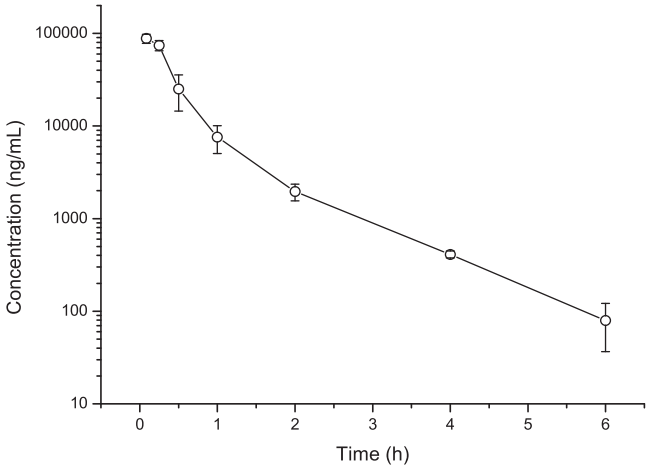

Fig. 3. The mean plasma concentration–time profiles of ASB after a single intravenous administration of ASB to dogs at a dose of 50 mg/kg ($n = 3$).

For the administration of ASB, pharmacokinetic parameter values of half-life ($t_{1/2}$), area under curve (AUC), systemic clearance (Cls), volume of distribution (V) and mean residence time (MRT) were 0.828 ± 0.114 h, $49{,}303 \pm 6740$ h ng/mL, 1.02 ± 0.13 L/h kg, 1.22 ± 0.23 L/kg and 0.504 ± 0.030 h, respectively.

4. Conclusion

A sensitive and reliable method was fully validated for the determination of ASB in dog plasma by LC–MS/MS. Good accuracy, precision and recovery were obtained and no significant matrix effect was observed. The method was successfully applied to measurement of ASB in dog plasma after a single intravenous administration of ASB to dogs at a dose of 50 mg/kg.

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