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Short communication

Determination of dehydroandrographolide succinate in human plasma by liquid chromatography tandem mass spectrometry (LC–MS/MS): Method development, validation and clinical pharmacokinetic study

Shuijun Li[a,b,1], Ding Yang[b,1], Mengqi Zhang[a], Juzhen Zhou[a], Rui Li[c], Chuan Lu[a], Yun Liu[a], Yanmei Liu[a], Yiping Wang[b], Chen Yu[a,*]

[a] Central Laboratory, Shanghai Xuhui Central Hospital, 966 Huaihai Middle Road, Shanghai 200031, China
[b] State Key Laboratory of Drug Research, Shanghai Institute of Materia Medica, Chinese Academy of Sciences, Shanghai 201203, China
[c] YaoPharma Co., Ltd., Chongqing 401121, China

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ABSTRACT

A LC–MS/MS method was developed and validated for the determination of dehydroandrographolide succinate (DAS), a traditional Chinese medicine derivative used for the treatment of pneumonia, upper respiratory tract infection, and chronic bronchitis. Following protein precipitation, DAS was detected by ion transition at *m/z* 531.2/99.0 in multiple reaction monitoring mode with negative electrospray ionization-tandem mass spectrometry. The limit of detection was 0.5 ng/mL, and the lower limit of quantification was 10 ng/mL in human plasma. Good linearity was maintained over the range of 10–5000 ng/mL, and the correlation coefficient was better than 0.99. The accuracy ranged from 95.3% to 113%, RSD from 0.928% to 6.47%, for the within- and between-run analysis at all QC levels. The recovery ranged from 85.5% to 93.4% and the matrix effect from 107% to 119%. No significant carryover and good stability were found during method validation. The developed method was successfully applied to the determination of DAS in human plasma in a pharmacokinetic study following intravenous infusion of potassium sodium DAS to nine healthy volunteers.

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1. Introduction

Dehydroandrographolide succinate (DAS, 14-deoxy-11,12-didehydroandrographolide-3, 19-disuccinate, Yanhuning) is a derivative of dehydroandrographolide, a major diterpenoid lactone found in the important traditional Chinese medicine, *Andrographis paniculata* Nees. DAS is generally salified with potassium or a combination of potassium and sodium salt and used as an injection formulation for the treatment of pneumonia, upper respiratory tract infection, and chronic bronchitis [1–3].

DAS is widely used in clinical practice in China. A sensitive and selective method for the pharmacokinetic or metabolism study of DAS in animal or human biological fluids is essential. However, only a few methods based on ultraviolet spectroscopy (UV) [4] or high performance liquid chromatography-ultraviolet detection (HPLC-UV) [5] have been reported for the determination of DAS in rabbit or rat plasma. These reported methods require 1.0 mL and 0.1 mL sample volume, and have sensitivity of 50 μg/mL and 50 ng/mL for the UV and HPLC-UV assay, respectively.

In this study, we have developed a liquid chromatography tandem mass spectrometry (LC–MS/MS) method, with the electrospray ionization source (ESI) operated in negative-ion mode, for the quantification of the drug DAS in human plasma. To the best of our knowledge, this is the first published method for DAS determination in human plasma with LC–MS/MS-based technology. This method is aimed at significantly enhancing sample throughput and reducing sample volume. After having been fully validated, it is applied to a pharmacokinetic study with a single dose of 160 mg potassium sodium DAS intravenously infused to nine healthy Chinese volunteers.

2. Experimental

2.1. Chemicals and reagents

Standard DAS (Fig. 1A) and the internal standard formononetin (IS, Fig. 1B), were purchased from the National Institute for the Control of Pharmaceutical and Biological Products, Beijing, China. HPLC-grade acetonitrile, methanol, and formic acid (96%) were

* Corresponding author. Tel.: +86 21 54037810x2130; fax: +86 21 54043676.
E-mail addresses: lishuijun@gmail.com (S. Li), cocozone@163.com (R. Li), ypwang@mail.shcnc.ac.cn (Y. Wang), chen-yu@online.sh.cn (C. Yu).
[1] These authors contributed equally to this work.

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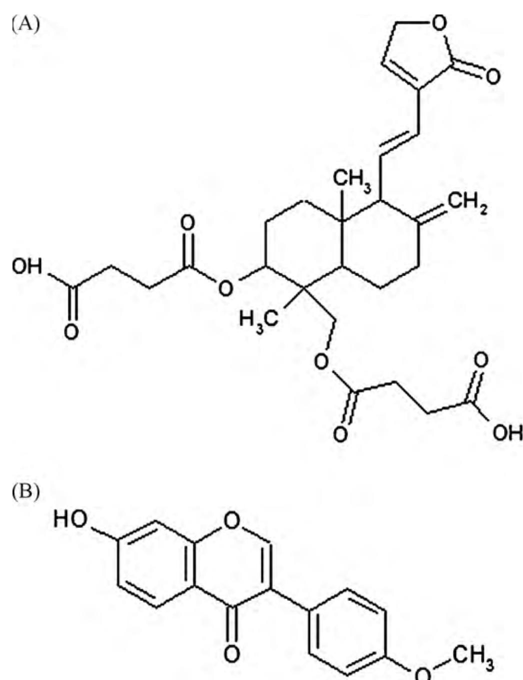

Fig. 1. Chemical structure of (A) DAS with the molecular formula $C_{28}H_{36}O_{10}$ and molecular weight 532.59 Da; and (B) formononetin (IS) with the molecular formula $C_{16}H_{12}O_4$ and molecular weight 268.26 Da.

purchased from Tedia Company, Inc. (Fairfield, OH, USA). Double distilled water was used throughout the study.

2.2. LC–MS/MS procedure

The Shimadzu liquid chromatography system (Kyoto, Japan) was equipped with two LC-20AD pumps, a SIL-20ACHT autosampler, an online DGU-20A3 vacuum degasser, and a CBM-20A control module. The chromatographic separation was achieved on a Shiseido Capcell C_{18} MG III column (100 mm × 2.0 mm i.d., 5 μm), preceded by a Phenomenex C_{18} guard column (4 mm × 3 mm i.d., 5 μm particle size). The mobile phase consisted of water and acetonitrile (58:42, v/v), containing 0.02% formic acid. It was pumped at an isocratic flow rate of 0.3 mL/min.

The liquid chromatography system was coupled to an AB SCIEX QTRAP 5500 mass spectrometer (Applied Biosystems, CA, USA), equipped with a Turbo V source and operated in a negative electrospray ionization mode. Data acquisition was performed in multiple reaction monitoring mode to record the transitions of the precursor ion to the product ion of DAS and IS. The mass spectrometry parameters were optimized by continuous infusion of 1 μg/mL standard solutions of DAS and IS. The optimal parameters of the ion source and compound included a −4.5 kV ion spray voltage, 550 °C source temperature, 0.4 MPa collision gas (pure nitrogen), 0.6 MPa curtain gas (pure nitrogen), 0.4 MPa nebulizer gas (pure nitrogen), and 0.6 MPa auxiliary gas (air gas). The optimal value of the declustering potential (DP), entrance potential (EP), collision energy (CE), and collision cell exit potential (CXP) was −70, −13, −35, −8 V for the DAS ion transition at m/z 531.2/99.0 and −60, −9, −30, −10 V for the internal standard formononetin ion transition at m/z 267.0/252.1 in multiple reaction monitoring scan, respectively. The resolution of Q1 and Q3 was set at unit, and the dwell time was selected as 200 ms. Analyst 1.5 software was used for instrument control, data acquisition, and quantification.

2.3. Standard solutions

Standard stock solutions were prepared by dissolving 2.04 mg DAS in 10 mL water/acetonitrile (50:50, v/v) and 1.64 mg IS in 10 mL methanol. IS working solutions (1 μg/mL) were obtained by diluting the stock solution with water/acetonitrile (20:80, v/v). All solutions were stored at 4 °C.

2.4. Sample preparation

To a 1.5 mL polypropylene tube, 50 μL human plasma, 5 μL IS working solution, and 100 μL precipitant (acetonitrile/methanol, 90:10, v/v) were mixed for 10 s and centrifuged at 15,000 × g for 3 min. A portion of the supernatant (50 μL) was diluted with 450 μL water/acetonitrile (60:40, v/v) in sample vials, and 2 μL was injected to LC–MS/MS.

2.5. Method validation

A full validation was performed by validating specificity, lower limit of quantification (LLOQ), linearity, accuracy, precision, carryover, recovery, matrix effect, and stability with respect to the recommended bioanalytical method validation by the Food and Drug Administration [6].

The method specificity was determined by analysis of six blank plasma from different subjects, with DAS concentrations spiked at 10, 350, 4000 ng/mL for six replicates. The spiked plasma were prepared and analyzed along with calibration curve to demonstrate the lack of endogenous interference and batch-to-batch variation.

The sensitivity of the analytical procedure was expressed as LLOQ or the lowest concentration on the calibration curve that can be quantitatively determined with acceptable accuracy within 80–12% and precision less than 20%. The signal to noise of DAS at LLOQ should be at least 10. The linearity of the method was evaluated by a calibration curve prepared in duplicate over a range of 10–5000 ng/mL DAS in plasma. A linear regression using fixed weighting factor was constructed based on the measured peak area ratio of DAS to the internal standard, versus the nominal concentration. The linearity was considered acceptable when the correlation coefficient (r) was higher than 0.99.

Precision (expressed by RSD for replicate measurements) and accuracy (expressed by the percentage of bias between nominal and calculated concentrations) were evaluated by analysis of six replicates of QCs at four concentration levels (10, 30, 350 and 4000 ng/mL) for three successive batches.

The carryover was evaluated by injecting a blank sample immediately after the upper limit of quantification (ULOQ) of the standard curve. The acceptance criterion of the carryover is the peak area of DAS in the blank sample less than 20% of that in a LLOQ [7]. The recovery was evaluated by comparing the peak areas in protein precipitated QC samples to those obtained in protein precipitated blank samples spiked with DAS and IS chemicals at three levels. The matrix effect was evaluated by comparing the peak areas in protein precipitated blank samples spiked with DAS and IS chemicals to those obtained in neat standard solutions at three levels.

The stability of DAS was investigated by subjecting QC samples to various conditions. Short-term bench top stability was evaluated by placing QC samples on the bench top at room temperature for 24 h. For freeze–thaw stability, QC plasma samples were subjected to three cycles from −30 °C to room temperature. The autosampler stability was assessed by placing processed QC samples in autosampler at 4 °C for 36 h. Long-term stability was evaluated by freezing QC samples at −30 °C for 4 weeks. The stock solution stability was determined by placing the stock solution mixture at −4 °C for 2

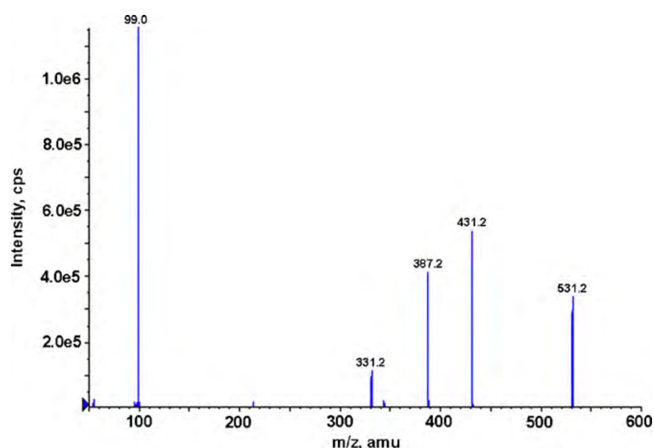

Fig. 2. Product ion spectra of DAS in the negative electrospray ionization mode.

months. The analytical results were compared with those of freshly prepared samples.

2.6. Method application

Nine healthy volunteers (six males and three females with age of 21–29 years) were recruited in the Phase I Clinical Research Center, Shanghai Xuhui Central Hospital, Shanghai, China. A physical examination was performed, and written informed consent forms were obtained before initiation of the pharmacokinetic study. The route, dose and time of administration of the study were defined by simulation the clinical application of DAS in the study protocol. A dose of 160 mg potassium sodium DAS was dissolved in 250 mL of 5% glucose solution and intravenously infused to volunteers at a constant rate over 1 h. A total of 3 mL of blood sample was drawn from the vein into heparinized tubes before dosing and at 0.167, 0.33, 0.5, 1, 1.17, 1.33, 1.5, 2.0, 2.5, 3.0, 4.0, 6.0, 8.0, 10.0, and 12.0 h after dosing. Plasma was separated by centrifugation at 3000 × *g* for 5 min, and it was stored at −30 °C until analysis. The study protocol and informed consent forms were approved by the ethics committee of the Shanghai Xuhui Central Hospital.

3. Results and discussion

3.1. Method development

To determine the scan polarity and optimize the mass spectrometric parameters for quantitative analysis, a standard solution of DAS with a concentration of 1 μg/mL in acetonitrile/water (50:50, v/v) was directly infused into the mass spectrometer at a continuous flow rate of 10 μL/min. Both negative and positive ion scan were investigated at the Q1 full scan mode. The negative electrospray ionization mode showed a significantly enhanced intensity than the positive mode with regard to the signal of the molecular ion of DAS detected in MS. Thus, it was selected for further study. Predominately deprotonated molecular ions $[M-H]^-$ at *m*/*z* 531.5 were observed at the negative electrospray full scan. The most abundant fragment ion was indentified using collision-induced dissociation at the product ion scan mode. Fig. 2 shows the fragmentation pattern and its corresponding product ions spectra from DAS, where the loss of 99 amu fragments dominated the pattern. The MRM mode was used for quantitative detection, with the most intense ion transition at *m*/*z* 531.2/99.0 for DAS.

The liquid chromatography conditions were also optimized during method development. Different types of analytical columns, several mobile phase combinations and elution program were tested. Ammonium acetate and formic acid were added in acetonitrile and water as mobile phases. As shown in Fig. 3A and B, by adoption a liquid chromatography condition with isocratic elution with mobile phase of 0.02% formic acid in water and 0.02% formic acid in acetonitrile (50:50, v/v) on Capcell C18 MG III (100 mm × 2.0 mm i.d., 5 μm, Shiseido), the intensity, peak shape, and the carryover greatly improved, with comparison to the condition of gradient elution with mobile phase of 5 mM ammonium acetate in water and acetonitrile on Allure PFP Propyl column (100 mm × 2.1 mm i.d., 5 μm, Resteck). As can be seen in Fig. 3C, compared with chloramphenicol, using formononetin as the IS and isocratic elution with 42:58 (v/v) acetonitrile/water (containing 0.02% formic acid) led to closer retention times between the IS and the analyte, avoiding being chromatographically interfered by the early eluted compounds.

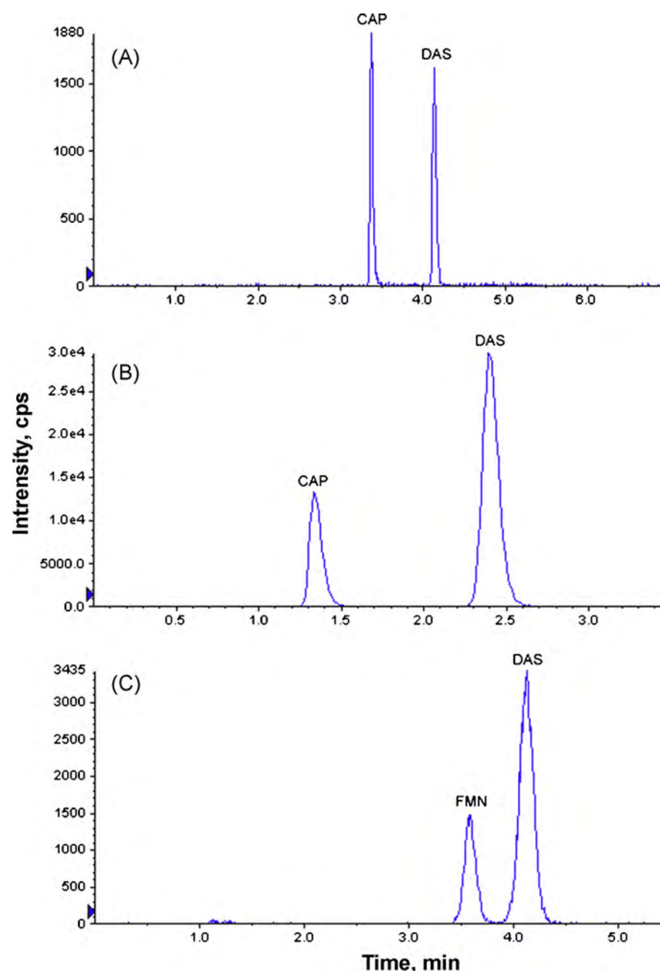

Fig. 3. Total ion chromatogram of DAS and the internal standard acquired from (A) gradient elution with mobile phase of 5 mM ammonium acetate in water and acetonitrile on Allure PFP Propyl column (100 mm × 2.1 mm i.d., 5 μm, Resteck), by using chloramphenicol as the internal standard, (B) isocratic elution with mobile phase of 0.02% formic acid in water and 0.02% formic acid in acetonitrile (50:50, v/v) on Capcell C18 MG III (100 mm × 2.0 mm i.d., 5 μm, Shiseido), by using chloramphenicol as internal standard, and (C) isocratic elution with ith mobile phase of 0.02% formic acid in water and 0.02% formic acid in acetonitrile (58:42, v/v) on Capcell C18 MG III (100 mm × 2.0 mm i.d., 5 μm, Shiseido), by using formononetin as the internal standard.

3.2. Method validation

3.2.1. Specificity, carryover, recovery, and matrix effect

The specificity was examined by comparing six different resources of blank human plasma with their corresponding spiked plasma at 30, 350, 4000 ng/mL concentrations of DAS. The accu-

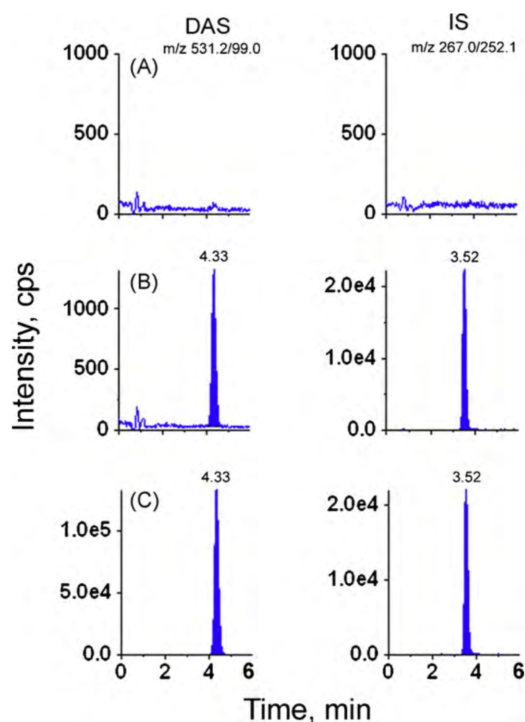

Fig. 4. Multiple reaction monitoring chromatograms of DAS (*m*/*z* 531.2/99.0) and IS (*m*/*z* 267.0/252.1) acquired from (A) blank human plasma, (B) blank plasma spiked with DAS at the LLOQ level (10 ng/mL), and (C) plasma collected 1 h after intravenous injection of 160 mg of potassium sodium DAS to a health volunteer.

racy of DAS spiked in six different resources of plasma ranged from 87.7% to 114.6% with an RSD of less than 5.27%, demonstrating similar results obtained from different sample resources. Fig. 4 presents the typical chromatograms of blank human plasma, blank plasma spiked with DAS at the LLOQ level (10 ng/mL), and plasma collected 1 h after intravenous infusion of 160 mg potassium sodium DAS to a healthy volunteer. As shown in the chromatograms, the baseline was flat, and no endogenous interference was observed at the retention time of DAS or IS. This indicates a good specificity for the method.

After the injection of each ULOQ of the standard curve, a blank sample immediately followed to evaluate the carryover. The DAS peak area in the blank was 3.21%, far less than 20%, of that in the LLOQ, and the IS peak area in the blank was 2.25% of that in the real sample. This shows that the carryover of the method was negligible.

To investigate the efficacy of the sample preparation, the recovery and matrix effects were evaluated by comparing the peak areas of DAS and IS between the three QC levels: 30, 350, 4000 ng/mL DAS spiked to blank plasma before sample preparation, to blank plasma after sample preparation, and pure standard solutions. Table 1 shows the results of the recovery and the matrix effect for DAS and IS. This was done by comparing the average peak areas for the six replicates, after they were prepared by protein precipitation. As can be seen in the table, the recovery ranged from 85.5% to 93.4% and the matrix effect from 107% to 119%. The results indicated satisfactory recovery and slight ion enhancement of the method. Before conducting the method validation, we also investigated the matrix effect by continuously post column infusion of a solution containing100 ng/mL of DAS and IS in mobile phase to mass spectrometry at a rate of 10 μL/min, and simultaneously injection of mobile phase, blank plasma (with protein precipitated) with an autosampler. No significant matrix effect was observed as no obvious signal perturbation occurs at the retention time of DAS and IS.

Table 1
Recovery and matrix effect of DAS and IS.

Analyte	DAS			IS
Nominal concentration (ng/mL)	30	350	4000	100
Recovery				
Mean (%)	85.5	93.4	92.1	89.9
RSD (%)	3.72	3.18	4.43	4.19
Matrix effect				
Mean (%)	119	107	109	111
RSD (%)	1.13	0.725	3.32	5.16

Recovery is evaluated by comparing the percentage of peak areas in protein precipitated QC samples to those obtained in protein precipitated blank samples spiked with DAS and IS chemicals. Matrix effect is evaluated by comparing the percentage of peak areas in protein precipitated blank samples spiked with DAS and IS chemicals to those obtained in neat standard solutions.

Table 2
Mean between-run calibration curve results of DAS in human plasma for 11 runs (with two replicates of seven concentration points per run).

Nominal concentration (ng/mL)	Mean concentration (ng/mL)	Bias (%)	RSD (%)
10	9.93	−0.73	2.91
20	20.2	1.03	4.02
100	102	2.02	3.44
500	506	1.18	2.47
1000	999	−0.132	3.38
2500	2480	−1.01	2.32
5000	4880	−2.49	2.61

Mean concentration is the average value of the calculated concentrations for all the runs. Bias (%) is the percentage of the difference between mean concentration and nominal concentration to the nominal concentration. RSD (%) is the percentage of standard deviation of calculated concentrations to average concentration.

3.2.2. Linearity and LLOQ

The linearity was evaluated by constructing seven-point calibration curves over the concentration range of 10–5000 ng/mL DAS in human plasma. This was done by calculating the peak area ratio of DAS to IS using a $1/x^2$ weighted linear regression method. Based on Table 2, the correlation coefficient (r) of the standard curve was better than 0.99 over a 500-fold concentration range for DAS with an overall relative standard deviation (RSD %) less than 4.02% and a bias within ±2.49% over 11 consecutive validation runs. To meet the requirements for the pharmacokinetics (PK) study, the LLOQ value must preferably be less than 5% of the maximum plasma concentration (C_{max}). Based on a signal-to-noise ratio (S/N) of no less than 3, the limit of detection (LOD) of this method was estimated to be lower than 0.5 ng/mL DAS in human plasma. As can be seen from Fig. 4B, the S/N value at LLOQ was obviously higher than 10. The LLOQ of the method was 10 ng/mL, at which level acceptable precision and accuracy were obtained (Table 3).

3.2.3. Precision and accuracy

The within- and between-run precision and accuracy of the developed method was assessed by determining QC samples at four different concentration levels (10, 30, 500, 4000 ng/mL), each with six replicates per run, for three consecutive runs. The QC samples were prepared and analyzed together with the calibration samples. Accuracy was expressed as the percentage of the mean value, from six determinations per concentration, to the theoretical value for DAS in the plasma. Precision was calculated as the %RSD of repeated determinations performed within a run or between runs. The results of within- and between-run precision and accuracy are summarized in Table 3. The within- and between-run accuracy ranged between 95.3% and 113% for all levels, including LLOQ. The RSD ranged from 0.928% to 6.47% for the within-run analysis and 2.66–5.45% for the between-run analysis. The results demon-

Table 3
Within- and between-run accuracy and precision of DAS in human plasma.

Nominal concentration (ng/mL)	Within-run						Between-run	
	Precision (%)			Accuracy (%)			Precision (%)	Accuracy (%)
	Run 1	Run 2	Run 3	Run 1	Run 2	Run 3		
10	6.47	5.48	3.93	95.3	99.4	97.3	5.36	97.3
30	2.65	2.25	3.09	102	102	113	5.45	106
350	2.60	2.26	0.928	97.7	104	103	3.33	102
4000	3.36	2.14	1.78	100	103	101	2.66	102
15000*	2.49	2.26	3.34	103	106	97.1	4.51	102
30000*		12.6			92.3		/	/

The within- and between-run precision and accuracy of the developed method is assessed by determining QC samples at four different concentration levels, each with six replicates per run, for three consecutive runs. Accuracy is expressed as the percentage of the mean value, from six determinations per concentration, to the theoretical value for DAS in the plasma. Precision is calculated as the RSD (%) of repeated determinations performed within a run or between runs.
* Diluting quality control.

Table 4
Stability of DAS in human plasma investigated at different conditions.

Nominal concentration (ng/mL)	Bias (%)	Accuracy (%)
Bench top stability (room temperature for 24 h)		
30	−8.02	88.9
350	−1.43	95.0
4000	−10.2	90.4
Freeze–thaw stability (three cycles from −30 °C to room temperature)		
30	7.19	104
350	5.78	102
4000	−0.523	100
Auto-sampler stability (4 °C for 36 post-preparation)		
30	−0.516	98.2
350	−3.03	98.6
4000	−1.31	98.5
Long-term storage stability (−30 °C for 4 weeks)		
30	5.02	102
350	11.6	108
4000	6.82	108

Bias (%) is calculated by concentrations at endpoint versus measured concentration at the start of the experiment. Accuracy (%) is calculated by concentrations at endpoint versus nominal concentration.

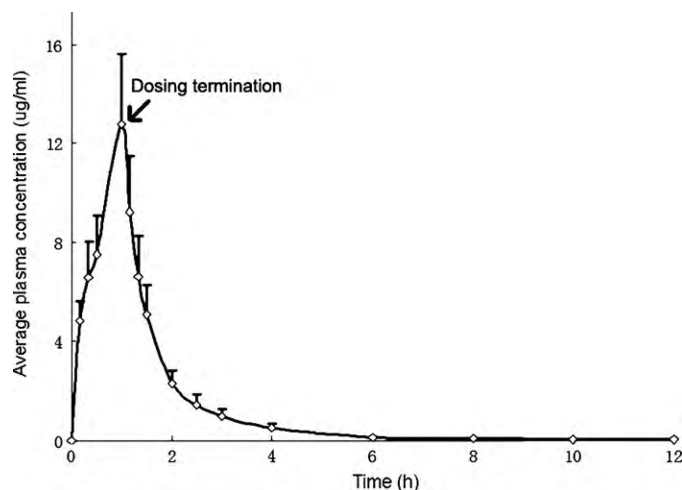

Fig. 5. Average plasma DAS concentration profile after intravenous infusion of a single dose of 160 mg potassium sodium DAS dissolved in 250 mL of 5% glucose solution to nine health volunteers.

strated that the values were within an acceptable criterion for the validated method.

Considering that the DAS concentration in some real samples may be higher than the upper limit of quantification due to the intravenous injection, we tested the dilution effect by diluting the 15,000 ng/mL QC sample 5-fold and diluting the 30,000 ng/mL sample 10-fold with blank human plasma. This resulted to 3000 ng/mL diluted QC sample. The diluted QCs were prepared as an unknown sample with six replicates. As shown in Table 3, the mean accuracies were 92.3–106%, and the RSD was less than 12.6%.

3.2.4. Stability

The stability was evaluated by subjecting the DAS QC samples to different conditions (Table 4). The short-term storage stability was assessed by maintaining low, middle, and high concentrations of QC samples on the bench top at room temperature for 0, 2, 4, 6, 24 h before analysis. The samples showed good stability at intervals investigated within a bias of ±10.2% compared to initial point (0 h). The freeze–thaw stability was determined after three freeze–thaw cycles from −30 °C to room temperature. The results showed that DAS was stable after going through three freeze–thaw cycles within a bias of ±7.19% compared to the initial point. By placing post-preparative QC samples in the autosampler at 4 °C for 6, 8, 24, and 36 h, DAS remained stable in human plasma within a bias of ±3.03% compared to the initial point (0 h). The long-term storage stability was evaluated by comparing the measured concentrations of the QCs stored at −30 °C for 1, 2, 3, and 4 weeks with those obtained before the storage period. The results indicated that no significant change (bias less than ±11.6%) was found after the storage period. We also investigated the stability of the stock solution by comparing the peak area of a DAS stock solution stored for two months at 4 °C with that of a freshly prepared solution. No degradation was observed during storage.

3.3. Clinical pharmacokinetic study

DAS is generally administered with intravenous infusion at 160–400 mg dose or intramascular injection at 40–80 mg dose per capita once daily in clinical practice. Till now, there is no study or data reported on the pharmacokinetics, distribution or metabolism of DAS, though its wide clinical practice in China. To better understand the clinical pharmacokinetics behaviors and the in vivo disposition of DAS, a sensitive LC–MS/MS method was developed to investigate its clinical pharmacokinetics in this study.

After being developed and validated, the method was applied to the determination of DAS concentrations in plasma after intravenous infusion of 160 mg potassium sodium DAS to nine healthy volunteers. The average plasma concentration versus the time profile of this drug is presented in Fig. 5. At the point (1 h) of dosing termination, DAS reached a maximum concentration, followed by a rapid clearance. The average $t_{1/2}$ was 1.8 h. Further research on

the elimination and metabolism of this drug is necessary in order to fully understand its in vivo distribution.

4. Conclusions

An analytical method based on liquid chromatography-negative electrospray ionization-tandem mass spectrometry method has been successfully developed for the determination of DAS in plasma. The methodology has been fully validated by assessing the standard stock solution, specificity, LLOQ, linearity, accuracy, precision, carryover, recovery, matrix effect, and stability. The method requires minimal sample preparation (50 μL plasma prepared by protein precipitation), allowing high throughput (6 min turnaround) and sensitive detection (LOD lower than 0.5 ng/mL with S/N > 3, LLOQ 10 ng/mL with S/N > 10). The validated method has been applied to the determination of DAS concentration in human plasma after intravenous infusion of 160 mg potassium sodium DAS to healthy volunteers in a clinical pharmacokinetic study.

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