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ORIGINAL ARTICLE

In vitro Metabolism of Sodium 9-dehydro-17-hydro-andrographolide-19-yl Sulfate in Rat Liver S9 by Liquid Chromatography–Mass Spectrometry Method

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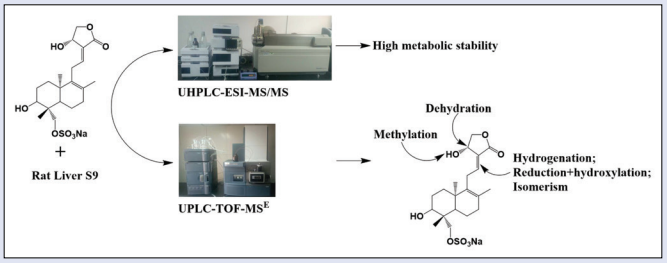
ABSTRACT

Background: Sodium 9-dehydro-17-hydro-andrographolide-19-yl sulfate (DHAS) is the active ingredient of Xiyanping injection, a traditional Chinese medicine in clinical use. However, there has been no report about the metabolic rate and metabolites of DHAS *in vitro*. **Materials and Methods:** In this article, DHAS was incubated with rat liver S9, and liquid chromatography/mass spectrometry (LC/MS) was used for the metabolism study. The residual concentrations of substrate were determined by ultra-high-performance liquid chromatography-electrospray ionization–tandem mass spectrometry method for the metabolic rate study of DHAS in liver S9. Metabolites were identified by the (UPLC-TOF-MS^E) Ultra-performance liquid chromatography/quadrupole time-of-flight mass spectrometry method. **Results:** The calibration curves of DHAS were linear over the concentration range from 0.75 μM to 75.22 μM with correlation coefficients >0.99. The lower limit of quantification was 0.150 μM for DHAS. The determination recoveries of DHAS were in the range of 84.9–90.6%. The $t_{½}$ and CL_{int} of DHAS in rat liver S9 were 98.6 ± 2.1 min and 3.5 ± 0.1 mL/min/g, respectively. Five metabolites were preliminarily identified based on the high resolution mass spectrum data in comparison with related references. These metabolites were mainly the products of dehydration and hydrogenation of DHAS. **Conclusion:** The present *in vitro* metabolic study of DHAS provided valuable information about the metabolic rate and potential metabolites of DHAS, which are important for future *in vivo* metabolism studies of DHAS and the discovery of more active andrographolide derivatives.

Key words: Liquid chromatography-mass spectrometry, metabolic rate, metabolites, rat liver S9, sodium 9-dehydro-17-hydro-andrographolide-19-yl sulfate

SUMMARY

- In this paper, sodium 9-dehydro-17-hydro-andrographolide-19-yl sulfate (DHAS) metabolism in vitro has been investigated with rat liver S9 using liquid chromatography-mass spectrometry (LC-MS). The result of quantitative analysis showed that DHAS had a long $t_{1/2}$, which indicated its high metabolic stability. Five metabolites of DHAS were identified in the incubation system based on the high resolution mass spectrum data in comparison with related references, particularly dehydrated and hydrogenated products. The results would provide certain references to screen out more active andrographolide derivative for pre-clinically.



Abbreviations used: MRM: Multiple reaction monitoring, DHAS: Sodium 9-dehydro-17-hydro-andrographolide-19-yl sulfate, IS: Internal standard.

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INTRODUCTION

Andrographolide is the main bioactive constituent of *Andrographis paniculata* Nees, a famous traditional Chinese and Indian medicine.[1,2] Andrographolide has demonstrated several biological activities, such as anti-inflammatory,[3,4] antibacterial,[5] antiviral,[6] and anticancer[7,8] activities. However, the poor solubility of this compound in water affects its bioavailability[9] and limits its use. Andrographolide sulfonate (trade name: Xiyanping injection), which is made by treating andrographolide with sulfuric acid,[10] has the effects of clearing heat and detoxifying, antibiosis, and enhancing the role of immunity.[11,12] Andrographolide sulfonate is highly water soluble and has been widely used for treating bronchitis, tonsillitis, and bacillary dysentery in China. Sodium 9-dehydro-17-hydro-andrographolide-19-yl sulfate [DHAS, Figure 1], one of the active ingredients of Xiyanping injection, was proved to have potent anti-microbial, anti-virus, and anti-inflammation activities in *in vitro* studies.[13,14] Now, the related report on the clinical application of DHAS indicated that DHAS had a shot $t_1/_2$ and distributed rapidly in plasma and tissue.[15] 9-dehydro-17-hydro-andrographolide (DHA), which is another active ingredient of Xiyanping injection, could be eliminated rapidly and was mainly metabolized to hydroxylated and dehydrogenated products in *in vitro* studies.[16] However, there is limited information available

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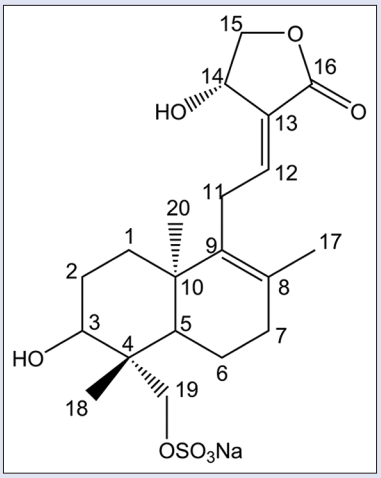

Figure 1: Chemical structure of sodium 9-dehydro-17-hydro-andrographolide-19-yl sulfate

about the *in vitro* metabolic rate and metabolites of DHAS, which has higher water solubility than DHA.

The *in vitro* model of metabolism based on the liver has been applied widely to study drug metabolism. Research methods have been reported including incubation in liver cell lines, liver microsome, liver S9, primary hepatocytes, etc., Incubation in liver S9 for drug is simple and cost-effective to get the data about drug metabolism, and the liver S9 contains rich enzymes.[17] Detailed study of drug metabolism is important to ensure that a drug can be used safely in humans. Liquid chromatography/mass spectrometry (LC/MS) is a progressive analytical tool to study drug metabolism.[18] LC/MS could determine within short time period and identify structures of the unknown analytes (metabolites) with high sensitivity and accuracy.

To acquire the optimal incubation conditions for DHAS, the ultra-high-performance liquid chromatography-electrospray ionization–tandem mass spectrometry (UHPLC-ESI-MS/MS) method was used to determine the residual concentrations of substrate for the metabolic rate study. In addition, UPLC-TOF-MS^E method was used to identify the metabolites of DHAS. Here, we report the preliminary data on the metabolic rate and the structures of metabolites for DHAS.

MATERIALS AND METHODS

Chemicals and reagents

DHAS (purity ≥99.0%) was obtained from Jiangxi Qingfeng Pharmaceutical Co. Ltd., (Ganzhou, China). Chloramphenicol was used as the internal standard (IS) and was purchased from the National Institute for Food and Drug Control (Beijing, China). Rat liver S9 was provided by CHI Scientific, Inc., (Jiangsu, China). The reduced form of nicotinamide adenine dinuclotide phosphate (NADPH), acetonitrile, and methanol (HPLC grade) were purchased from Sigma-Aldrich Inc., (Taufkirchen, Germany). Formic acid (MS grade) was purchased from Sigma-Aldrich Inc., (Taufkirchen, Germany). De-ionized water was generated from a Milli-Q-system (Millipore, Milford, MA, USA). All other reagents were of analytical grade.

Preparation of standard solutions and quality control samples

Stock standard solutions of DHAS (2.72 mg/mL) and IS (chloramphenicol, 110 ng/mL) were separately prepared by accurately weighing and dissolving in methanol. All solutions were stored at 4°C in the dark.

Quality control (QC) samples were prepared at three concentrations (QC low [QC-L], QC medium [QC-M], and QC high [QC-H]) for DHAS. These solutions were diluted in inactivated rat liver S9 to produce three QC levels from the stock standard solutions of DHAS. Subsequent steps were processed according to the sample preparation in incubation for quantitative analysis as described below. All QC samples were stored at −20°C in the dark for analysis.

Incubation for quantitative analysis

The incubation was performed in 0.1 M phosphate buffer (PH 7.4) and contained 50 μM DHAS, 2 mg/mL rat liver S9, and 1 mmol/L NADPH. Liver S9 incubates were being prewarmed for 5 min before the reaction was initiated by adding the NADPH. After incubation at 37°C for proper time in a shaking water bath, the reaction was stopped by adding an equal volume of ice-cold acetonitrile with IS. All samples were centrifuged at 13,000 rpm for 10 min, then 150 μL of the supernatant was transferred and dried under a gentle N_2 gas flow at 37°C. The residue was re-dissolved in 150 μL of 50% acetonitrile solution and centrifuged at 13,000 rpm for another 10 min. An aliquot of 1 μL supernatant was injected into the UHPLC-MS/MS system for analysis.

Instrumental conditions for quantitative analysis

The LC separations were performed on an Agilent 1290 series UHPLC system (Agilent Technologies, USA). A Waters ACQUITY UPLC® BEH-C_{18} column (2.1 mm × 100 mm, 1.7 μm) at 40°C was used to separate DHAS and IS with a mobile phase gradient of acetonitrile (A) and 0.01% formic acid in ultra-pure water (B): 10% A (0–1.0 min), 10–80% A (1.0–4.0 min), and 80–10% A (4.1–6.5 min). The total run time was 6.5 min at a flow rate of 0.4 mL/min and the injection volume was 1 μL.

The API 4000 triple-stage quadrupole mass spectrometer (AB Sciex, USA) equipped with a Turboionspray™ interface was detected using electrospray ionization (ESI) in the negative ionization mode, and the multiple reaction monitoring mode was used to quantify. The optimized conditions were as follows: Ion source gas 1 (GS 1), 60 psi; ion source gas 2 (GS 2), 60 psi; curtain gas, 25 psi; source temperature, 500°C; capillary voltage, −4500 V; entrance potential, −10 V; and collision cell exit potential, −11 V. The precursor–product ion pairs were: m/z 428.9→96.0 (DHAS) and m/z 320.9→151.8 (chloramphenicol, IS). The de-clustering potential and collision energy were −80 and −80 eV for DHAS and −71 and −23 eV for IS. The instruments controlled and data collected were acquired using Analyst® software (version 1.5.2, Applied Biosystems, CA, USA).

Method validation

The validation of this method was performed according to the guidelines set by the European Medicines Agency for bioanalytical method validation.[19]

The calibration curves of DHAS were defined by eight concentrations from 0.75 to 75.22 μM and constructed by plotting peak-area ratios of DHAS to IS against the concentrations of DHAS of least square linear regression analysis with a weighting factor of $1/x^2$. The lower limit of quantification (LLOQ) is the lowest concentration that could be measured with the precision <20% and accuracy within ± 20%.

The specificity was assessed by comparing chromatograms of blank rat liver S9, blank rat liver S9 spiked with DHAS, and rat liver S9 after incubation for 120 min.

The precision and accuracy were determined by analyzing DHAS at the QC-L, QC-M, and QC-H concentrations. Intra-day precision and accuracy were determined by using six replicates in a day. Inter-day

precision and accuracy were tested in 3 consecutive days. Precision was calculated as the relative standard deviation (RSD, %) and accuracy was expressed as the relative error (RE, %).

The extraction recovery of DHAS was calculated by the areas of QC samples against the standard solution. The matrix effect was evaluated by comparing the mean peak areas of DHAS re-constituted incubation at three different concentrations with that of the standard solution.[20]

The stability of DHAS in rat liver S9 was evaluated at three QC levels under a variety of conditions. Short-term stability was assessed under room temperature (25°C) at 0, 2, 4, 8, 12, and 24 h, respectively. Autosampler stability was evaluated after 12 h storage of samples ready for injection in the autosampler at 4°C. Freeze–thaw stability was determined after three freeze–thaw cycles. And, the sample was considered stable when the RSD was within ± 15%.

Metabolic rate study

Incubation time was investigated from 0 to 300 min (0, 15, 30, 60, 90, 120, 180, 240, and 300 min) (n = 3). The liver S9 protein concentration was evaluated from 0.2 to 2.0 mg protein per mL of incubation medium (0.2, 0.4, 0.8, 1.0, 1.5, and 2.0 mg/mL) (n = 3). To stop the metabolic reaction, ice-cold acetonitrile with IS was added. The remaining concentration of DHAS was calculated by the calibration curve. Moreover, the percentage of remaining DHAS was obtained by the residual concentration compared with that of DHAS at 0 min. The slope of the linear regression, which was constructed by plotting the log percentage remaining versus incubation time, was used to calculate the $t_{½}$. The *in vitro* $t_{½}$ and CL_{int} of DHAS in the liver S9 were calculated according to the following formula: $t_{½} = -0.693/k$, $CL_{int} = 0.693/t_{½}$ (min) × incubation (mL)/incubation (mg).[21]

Incubation for qualitative analysis

The incubation is the same as the incubation for quantitative analysis, but the incubation system expanded 5 times. To stop the reaction, an equal volume of ice-cold acetonitrile without IS was added after incubation for 240 min at 37°C. The mixture was centrifuged at 13,000 rpm for 10 min, and the supernatant was dried under a gentle N_2 gas flow at 37°C. The residue was re-dissolved in 1 mL of water and centrifuged at 13,000 rpm for another 10 min. The supernatant was successively washed with 2 mL of water and 2 mL of 30% methanol solution on an *AGT* Cleanert™ C_{18} solid-phase extraction (SPE) (200 mg/3 mL). And, collect the eluent of 30% methanol solution. The eluent was dried and re-dissolved in 1 mL of 50% acetonitrile solution.

Instrumental conditions for qualitative analysis

The separation was performed on a Waters ACQUITY UPLC® BEH-C_{18} column (2.1 mm × 100 mm, 1.7 μm) with the column temperature at 40°C. The mobile phases were acetonitrile (A) and 0.1% formic acid in ultra-pure (B) eluting in a gradient program: 0–5.0 min, linear from 5% to 50% A; 5.0–5.2 min, linear from 50% to 90% A; 5.2–7.0 min, and linear from 90% to 5% A; 7.0–8.0 min 5% A for equilibration of the column. The injection volume was 3 μL.

Mass spectrometric detection was performed on a Waters ACQUITY™ Synapt TOF mass spectrometer (Waters Corp., Manchester, UK) connected with a UPLC system via an ESI interface. The ESI source was operated in a negative ion mode for recording the mass spectra, and leucine-enkephalin was used as the calibration solution. The acquisition parameters for negative modes were: Sampling interval, 0.3 s; mass range recorded m/z, 70 ~ 600; capillary voltage, 3.5 kV; cone voltage, 35 kV; source temperature, 150°C; de-solvation temperature, 500°C; de-solvation gas (N_2) flows, 1000 L/h; cone (N_2) flows, 50 L/h; collision gas, argon; low energy collision, 6 eV; and high energy collision, 15~45 eV.

RESULTS AND DISCUSSION

Chromatography conditions and mass spectrometry/mass spectrometry optimization

Compared to conventional HPLC, the LC/MS with high resolution and specificity was chosen to determine the concentration of substrate and IS. Three mobile phases (acetonitrile–water, acetonitrile–methanol–water, methanol–water) were tested in this study. However, peak-tailing of chromatographic peak shapes was found in all mobile phases mentioned for DHAS. To improve the peak shape, different concentrations of formic acid in water phase were studied. We found that a good response and peak shape for DHAS were displayed in the acetonitrile and 0.01% formic acid solution. Both the positive and the negative ion modes were tested for the analyte and IS. The results showed that DHAS and IS in negative mode had a higher response than that in positive mode. Hence, we selected the negative ion mode for the ionization. According to the level of full scan results, 428.9 (M-Na) and 320.9 (M-H) were selected as parent ions for DHAS and IS, respectively. The product ions at m/z 96 and m/z 151.8 were the quantitative ions of DHAS and IS, respectively.

Preparation of samples

Acetonitrile could effectively remove proteins by comparing other kinds of organic solvents. Therefore, acetonitrile was selected to remove proteins for quantitative analysis in this study. However, large polar impurities still existed in the sample after de-proteinization and caused higher response than metabolites in full-scan MS detection. Eluting

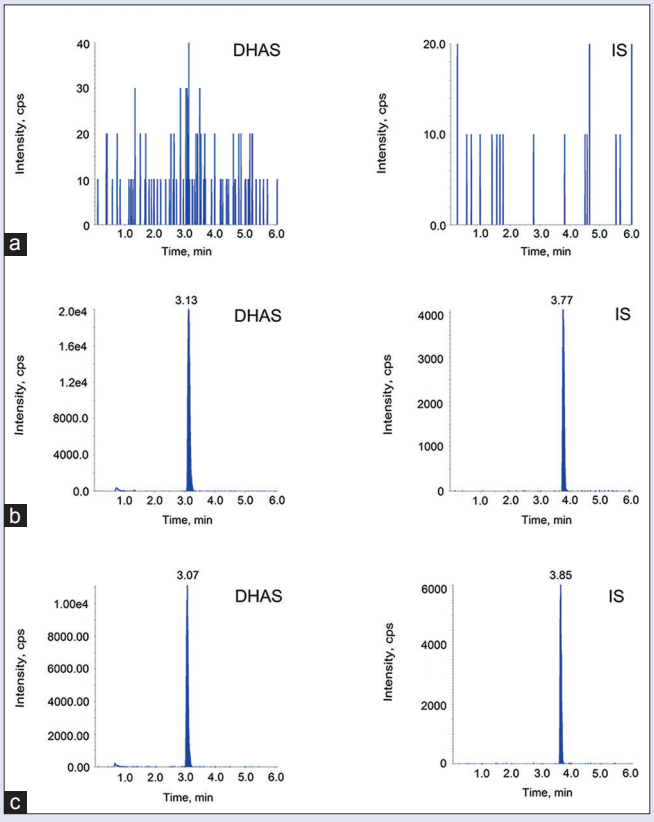

Figure 2: Representative multiple reaction monitoring chromatograms of sodium 9-dehydro-17-hydro-andrographolide-19-yl sulfate (428.9→96.0) and IS (320.9→151.8) for blank rat liver S9 (a), blank rat liver S9 spiked with sodium 9-dehydro-17-hydro-andrographolide-19-yl sulfate and internal standard (b), incubated at 37°C for 120 min in rat liver S9 (c)

on C_{18} SPE for further removing large polar impurities could enhance the response of metabolites. Hence, SPE was used to prepare biological samples for qualitative analysis.

Method validation results

The selectivity assay showed no interfering peaks at the retention of the DHAS or the IS from the biological matrix [Figure 2].

The calibration curve for DHAS was linear over the concentration of 0.75–75.22 μM. Moreover, the regression equation was $y = 0.12x + 0.0684$ ($R^2 = 0.9918$), where y is the peak-area ratios of DHAS to the IS, x is the respective concentration. The LLOQ was 0.150 μM for DHAS, so the method is sensitive for DHAS in qualitative analysis.

The intra-day and inter-day precisions and accuracies of DHAS are summarized in Table 1. The intra-day and inter-day precisions (RSD) ranged from 1.9% to 6.3%, at three concentration levels of 0.75, 30.09, and 60.18 μM, respectively. The RE values for intra-day accuracy and inter-day accuracy were within ± 10% for three QC levels, respectively. The result indicates that our method is accurate and precise to meet the guidelines for bio-analytical methods.

Table 2 summarizes the matrix effect and recovery data for DHAS. The extraction recovery and matrix effect of DHAS ranged from 84.9% to 90.6% with RSD <5.2% and 94.6% to 98.9% with RSD <4.0% at three concentration levels of 0.75, 30.09, and 60.18 μM, respectively. The results indicate that the sample preparation method used in the present assay was acceptable and no significant matrix effects were observed on the DHAS and IS in this study.

The result showed that DHAS was stable in PB solution and inactive rat liver S9 under room temperature for 0–24 h (RE: −4.3–0.2%, RSD <5.1%) in the autosampler for 12 h (RE: −3.5–0.8%, RSD <4.8%), during three freeze–thaw cycles and at −20°C for 6 weeks (RE: −6.5–1.5%, RSD <6.1%). Hence, the DHAS was considered stable during the sample preparation and chromatographic measurements.

Optimization of incubation conditions and metabolic rate study

The rat liver S9 protein concentration was varied from 0 to 2.0 mg/mL and percent conversion gradually stabilized under 2.0 mg/mL of protein concentration. The incubation time was varied from 0 to 300 min, and the result showed that a good linearity was observed in the range of 0–120 min and the concentration of DHAS did not change in 240 min. Based on the above results, 2 mg/mL was chosen as the protein concentration. 120 min was selected as the incubation time for quantitative analysis. And, 240 min with larger percent conversion was selected as the incubation time for qualitative analysis. Figure 3 shows the percent conversion change of DHAS with time. The mean percent conversion of DHAS was 83.3% within 240 min. The $t_{½}$ and CL_{int} of DHAS in rat liver S9 were 98.6 ± 2.1 min and 3.5 ± 0.1 mL/min/g protein, respectively.

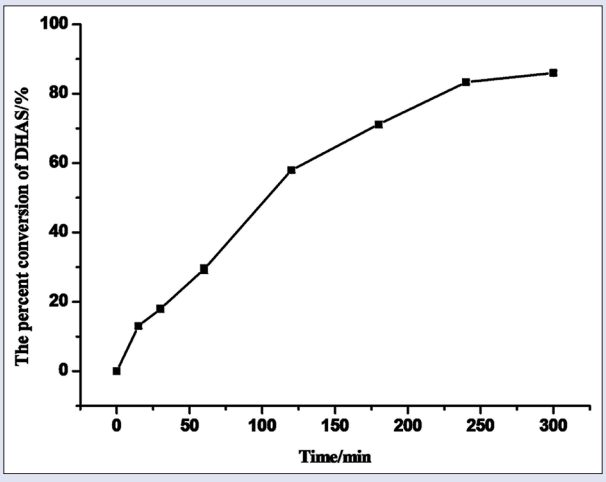

Figure 3: Effect of incubation time on the percent conversion change of sodium 9-dehydro-17-hydro-andrographolide-19-yl sulfate in rat liver S9

Analysis of metabolic *in vitro*

Blank liver S9 spiked with DHAS, DHAS after incubation for 240 min in liver S9, and blank liver S9 were scanned in negative ion mode by the UPLC-TOF-MS^E [Figure 4]. Five unknown peaks were found in the chromatogram and they might be the metabolites of DHAS in liver S9.

Probable structures were considered in accordance with the metabolic rules of andrographolide and structural characteristics of DHAS to identify the metabolites *in vitro*. Table 3 shows the retention time, characteristic metabolic reaction, experimental mass m/z, elemental composition, and fractional mass difference in the MS/MS stage of target metabolites. Five

Table 1: Precision and accuracy of the assay method for sodium 9-dehydro-17-hydro-andrographolide-19-yl sulfate in rat liver S9 (n=6)

Analytes	Nominal concentrations (μM)	Intra-day precision			Inter-day precision		
		Measured concentration (mean±SD)	RSD (%)	RE (%)	Measured concentration (mean±SD)	RSD (%)	RE (%)
DHAS	0.75	0.76±0.04	5.6	0.4	0.75±0.04	4.7	−0.3
	30.09	29.62±0.92	3.1	−1.5	30.13±1.9	6.3	0.2
	60.18	56.42±1.08	1.9	−6.3	57.63±1.40	2.4	−4.2

SD: Standard deviation; RSD: Relative standard deviation; DHAS: Sodium 9-dehydro-17-hydro-andrographolide-19-yl sulfate; RE: Relative error

Table 2: Matrix effect and recovery of the assay method for sodium 9-dehydro-17-hydro-andrographolide-19-yl sulfate in rat liver S9 (n=6)

Analytes	Nominal concentrations (μM)	Extraction recovery (%)		Matrix effect (%)	
		Mean±SD	RSD	Mean±SD	RSD
DHAS	0.75	90.6±4.7	5.2	98.9±3.9	4.0
	30.09	85.7±3.6	4.2	94.6±1.8	1.9
	60.18	84.9±2.4	2.8	96.7±3.1	3.3

SD: Standard deviation; RSD: Relative standard deviation; DHAS: Sodium 9-dehydro-17-hydro-andrographolide-19-yl sulfate

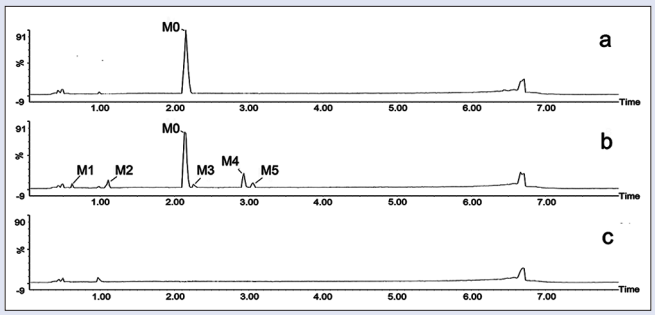

Figure 4: UPLC-photodiode array chromatograms of blank liver S9 spiked with sodium 9-dehydro-17-hydro-andrographolide-19-yl sulfate (a), sodium 9-dehydro-17-hydro-andrographolide-19-yl sulfate after incubation of 240 min in liver S9 (b) and blank liver S9 (c)

probable metabolites were analyzed according to the precise molecular weight and information of fragment ion MS^2 of each metabolite and the related reference for biotransformation of andrographolide. Possible metabolic pathways and structure of metabolites were inferred for DHAS in liver S9. The inferences are as follows:

Under the function of ESI source, the m/z 429 of M0 produced fragment ions of m/z 411, m/z 315, and m/z 96 after initial collision and induced dissociation. The m/z 411 was generated by losing 18 (H_2O) and the further loss 14 (-CH_2) produced the m/z 397. The m/z 313 was originated by the break of 19 C-O bond and the diterpene's bicyclo losing 2 (H_2) from the m/z 411. The m/z 315 formed by losing 2 (H_2) after the break of C-C between 11 and 12 from the m/z 429. The further losing 14 (-CH_2) of the m/z 315 produced fragment ions of m/z 301. The fragment ion at m/z 96 was formed by cleavage of 19 C-O bond from the m/z 429. Proposed fragmentation pathways of DHAS were presented in Figure 5. Figure 6 shows MS^2 Spectra of compounds M0~M5.

The characteristic molecular ion of M1 (retention time 0.63 min) was obvious at m/z = 447. The m/z 387 was originated from the cleavage of C-C bond between 13 and 14, losing 18 (H_2O) in 12 and 16 ester bond hydrolysis from the m/z 447. The m/z 327 was formed from the break of C=C bond between 12 and 13 combining with the diterpene's bicyclo losing 2 (H_2) from the m/z 387. The fragment ion at m/z 403 was generated by the m/z 447 losing 44 (-COO) in the five-membered lactone. The forming paths of m/z 315 and 96 were similar to the fragment ions in M0. Combining with the relevant reference,[22] reduction and hydroxylation could occur in the C=C bond between 12 and 13 of andrographolide. Therefore, M1 could be formed through reduction and hydroxylation of DHAS.

The base peak of M2 (retention time 1.12 min) in the MS/MS spectrum m/z 431 produced the m/z 413 and m/z 429 by loss of 18 (H_2O) and 2 (H_2), respectively. The forming paths of m/z 411, m/z 315, m/z 301, and m/z 96 were similar to the fragment ions in M0. Combining with the relevant reference,[23] the double bond between 12 and 13 of andrographolide could easily hydrogenate. Hence, M2 was inferred to be from hydrogenation of DHAS.

Metabolite M3 has a similar retention time (2.26 min) and the same base peak with M0. The forming paths of m/z 411, m/z 397, m/z 315, and m/z 96 were similar to the fragment ions in M0. Combining with the relevant reference,[22] spatial isomerization combining the dehydration reaction could easily occur after the double bond between 12 and 13 of andrographolide. Therefore, M3 was inferred to be the isomer of DHAS.

Table 3: Information of sodium 9-dehydro-17-hydro-andrographolide-19-yl sulfate and its metabolites

Compound	Retention time (min)	Metabolic reaction	m/z	Elemental composition	Fractional mass difference/mDa
M0	2.16	Null	429.1575	$C_{20}H_{29}O_8SNa$	−0.8
M1	0.63	Reduction + hydroxylation	447.1711	$C_{20}H_{31}O_9SNa$	2.2
M2	1.12	Hydrogenation	431.1740	$C_{20}H_{31}O_8SNa$	0
M3	2.26	Isomerism	429.1597	$C_{20}H_{29}O_8SNa$	1.4
M4	2.96	Dehydration	411.1458	$C_{20}H_{27}O_7SNa$	−1.9
M5	3.10	Methylation	443.1731	$C_{21}H_{31}O_8SNa$	−0.9

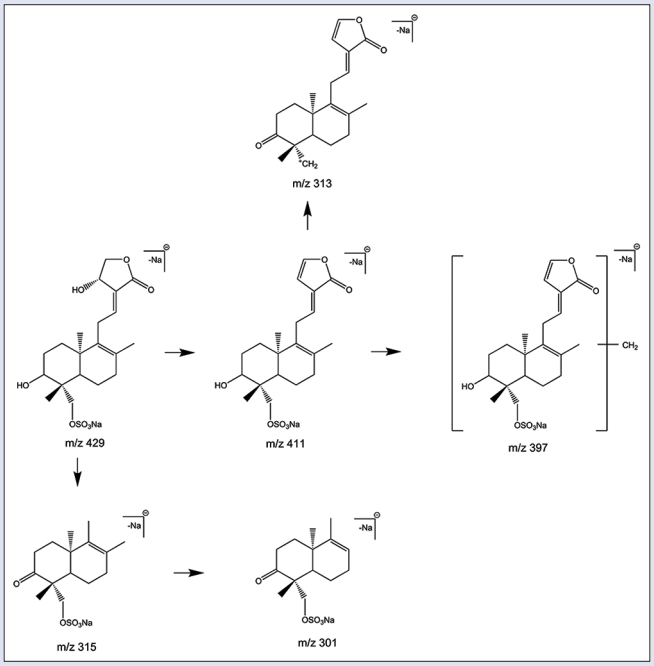

Figure 5: Proposed fragmentation pathways of sodium 9-dehydro-17-hydro-andrographolide-19-yl sulfate

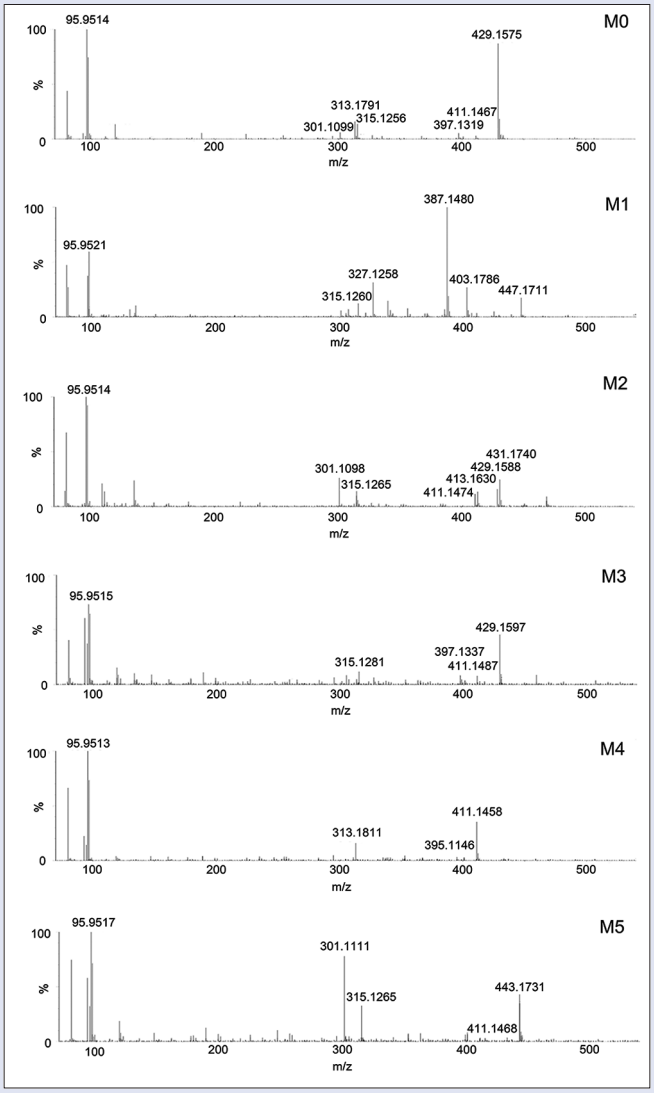

Figure 6: MS^2 spectra of compounds M0~M5

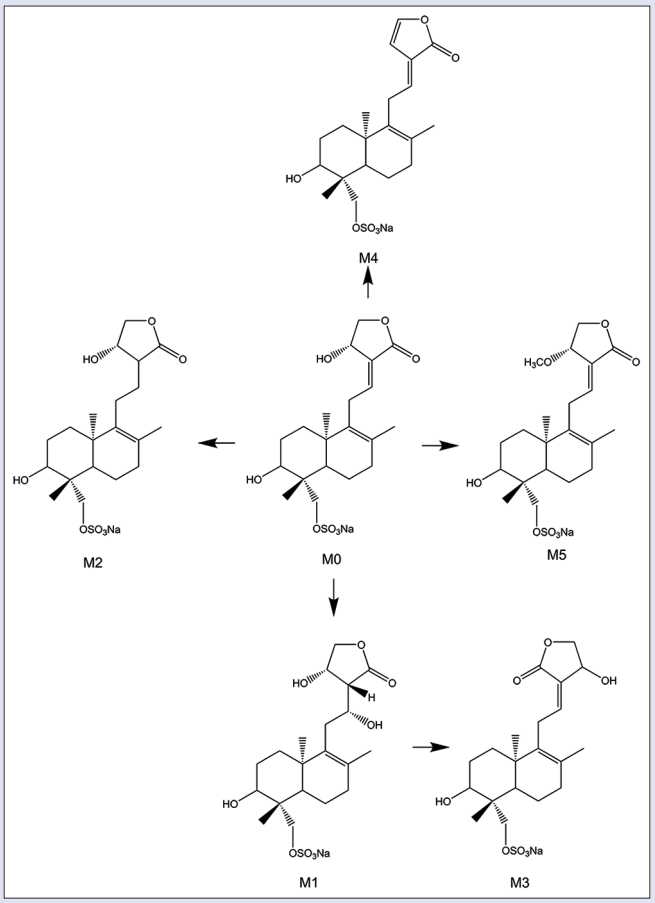

Figure 7: Possible metabolic pathways of sodium 9-dehydro-17-hydro-andrographolide-19-yl sulfate in rat liver S9

The base peak of M4 (retention time 2.96 min) was m/z 411. The m/z 395 was generated by losing 16 of the fragment ion at m/z 411. The m/z 313 was originated by the break of 19 C-O bond and the diterpene's bicyclo losing 2 (H_2) from the m/z 411. The m/z 313 was formed from the cleavage of C-O bond in 19 and the diterpene's bicyclo losing 2 (H_2) of m/z 411. The forming path of m/z 96 was similar to the fragment ion in M0. Combining with the relevant reference,[24] the five-membered lactone of andrographolide could easily dehydrate. Hence, M4 was the dehydration product of DHAS.

Metabolite M5 was detected at 3.10 min, which indicated that the polarity of M5 decreased, and the molecular ion peak was observed at m/z 443, and further loss 32 produced the m/z 411. The fragment ion at m/z 315 was produced by the diterpene's bicyclo losing 2 (H_2) after the break of C-C bond between 11 and 12 from the m/z 443, and further loss 14 (-CH_2) formed the m/z 301. The forming path of m/z 96 was similar to the fragment ion in M0. Combining with the relevant reference,[23] the hydroxyl in 14 of andrographolide could easily be methylated. Therefore, M5 was inferred to be the methylation product of DHAS.

We proposed the possible metabolic pathways of DHAS in rat liver S9 according to the above results [Figure 7].

CONCLUSIONS

The LC–MS/MS method developed in the present study is sensitive and reliable for the determination of DHAS in rat liver S9. The method has been successfully applied to the metabolic rate study of DHAS *in vitro*. The data clearly show that DHAS was metabolized in rat liver S9 and provide the best incubation time and S9 concentration. According to the results, DHAS had a long $t_{1/2}$, which indicated its high metabolic stability. The result of qualitative analysis showed that the major products of DHAS in rat liver S9 were the dehydrated and hydrogenated metabolites. It is unclear whether any of the metabolites of andrographolide proposed in this study exhibits the reported biological activities of androgrpholide itself. Further research is warranted in investigationg the activity of these metabolites, which may lead to the discovery of more active andrographolide derivative.

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Conflicts of interest

There are no conflicts of interest.

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