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*Research Article*

# Rapid Analysis of Aristolochic Acid Analogues in Traditional Chinese Patent Medicine by LC-MS/MS

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Aristolochic acids have been demonstrated to have renal toxicity, cause carcinogenesis, and may cause gene mutations. A series of risk control measurements have been adopted worldwide since 1990s. Some varieties of traditional Chinese medicine with high content of aristolochic acids have been banned in China. However, some species containing aristolochic acids in microscale are still in use. In recent years, with the continuous awareness of drug safety, the aristolochic acid analogues were generally considered to be of potential safety risks. Among these constituents, aristolochic acid I is still the one with most studies. Therefore, in addition to aristolochic acid I, it is necessary to establish an accurate and rapid method to determine other aristolochic acid analogues. LC-MS/MS methods based on multireaction monitoring mode was established to simultaneously determine 9 aristolochic acid analogues including 5 aristolochic acids and 4 aristolactams for the first time. Furthermore, the method was applied for Long dan Xie gan Pill, a traditional complex compound preparation with a long history for treatment of diseases including hepatochlic hygropyrexia, dizziness, tinnitus, and deafness. It has attracted widespread attention because of the aristolochic acid nephropathy. The crude drug Caulis Aristolochiae manshuriensis (Guanmutong) collected in the prescription was replaced by Akebiae Caulis (Mutong), and the established method helps to understand the product safety on market. As a result, aristolochic acid I, aristolochic acid Iva, and aristolactam I were detected and determined in one batch of Long dan Xie gan Pill among 25 batches of samples. It provided practical approach to demonstrate trace aristolochic acids and aristolactams. It was beneficial to control the safety of related traditional Chinese medicine products.

## 1. Introduction

Aristolochic acid (AA) analogues are a kind of compounds with nitrophenanthrene core structure, including aristolochic acids and aristolactams (ALs). These compounds mainly exist in the genus of *Aristolochia* and *Asarum* from the family of Aristolochiaceae plants. It has been proved that some aristolochic acids have renal toxicity, cause carcinogenesis, and may cause gene mutations [1–6]. Thus, a series of risk control measurements have been adopted worldwide since 1990s [7–9]. Three varieties of Caulis Aristolochiae manshuriensis, Radix Aristolochia fangchi, and Radix Aristolochiae with high content of aristolochic acids have been banned in China since 2003. However, some Chinese herbal medicines and preparations with trace amount of aristolochic acids are still in use. Until now, most of the toxic studies are focused on AA I. Due to the serious adverse effect of AAs and the insufficient research on other aristolochic acid analogues except for AA I, it is essential to develop a simple and fast method to identify and quantify the AA analogues in commonly-used products. Since the structures of these AA analogues are similar and some are isomers with different substitution positions of hydroxyl and/or methoxy groups, it is challenging to qualify and quantify the AA analogues at the same time.

At present, different methods have been reported for determination of AAs, including thin-layer chromatography (TLC), high performance liquid chromatography (HPLC), liquid chromatography-mass spectrometry (LC-MS), enzyme-linked immunosorbent assay (ELISA), and capillary electrophoresis (CE) [9–11]. The HPLC and LC-MS methods are also widely used in characterizing the AA analogues [12–18]. HPLC

is more suitable for those with higher content of AA analogues. For traditional Chinese patent medicines with trace amount of AA analogues or with component interference, LC-MS shows strong advantages of high specificity and high sensitivity. LC-MS has been applied for qualification by MS and MS/MS spectra comparison. Meanwhile, it is also used for quantification mainly based on the extracted ion chromatogram (EIC) mode. Also, the reported multireaction monitoring (MRM) mode was mainly focused on AA I and/or AA II. In this study, we report that the new LC-MS/MS method is capable of characterizing 9 AA analogues (Figure 1) including aristolochic acid I, aristolochic acid II, aristolochic acid IIIa, aristolochic acid IVa, 7-hydroxy aristolochic acid I, aristolactam I, aristolactam AIIIa, aristolactam BII, and aristolactam FI for the first time.

Moreover, the LC-MS/MS method was further applied to Long dan Xie gan Pill, a commonly-used traditional Chinese patent medicine, with a long history for treatment of diseases including hepatochlic hygropyrexia, dizziness, tinnitus, and deafness [19, 20]. Long dan Xie gan Pill has attracted widespread attention since it caused aristolochic acid nephropathy (AAN) by Caulis Aristolochiae manshuriensis (Guanmutong) which existed in the prescription before 2003 [21]. Then, toxic Guanmutong was replaced by Akebiae Caulis (Mutong) without AAs inside. In order to understand whether there is still safety risk of raw material adulteration and further investigate the product safety, 25 batches of Long dan Xie gan Pills from 8 enterprises were analyzed. The results indicated that the established method could efficiently analyze the aristolochic acid analogues for qualitative and quantitative purposes. Also, it could provide a practical approach for related species, especially traditional Chinese patent medicines containing trace content of aristolochic acid analogue constituents. The LC-MS/MS method reported in this paper was valuable for the safety control of related traditional Chinese medicine products.

## 2. Materials and Methods

*2.1. Chemicals and Reagents.* AA I (99.1%, Lot no. 110746–201912) was from National Institutes for Food and Drug Control, Beijing, China. AA II (HPLC purity≥ 98%, Lot no. P13J10F90613), AA IIIa (HPLC purity≥ 98%, Lot no. P20N8F48641), AA IVa (HPLC purity≥ 98%, Lot no. Z03J10X89866), 7-hydroxy AAI (HPLC purity≥ 95%, Lot No. Z13N9S74959), AL I (HPLC purity≥ 98%, Lot no. P27N10S104067), AL AIIIa (HPLC purity≥ 98%, Lot no. T09M11Z112630), AL BII (HPLC purity≥ 97%, Lot no. X09M11_112631), and AL FI (HPLC purity≥ 98%, Lot no. X09M11L112632) were from Shanghai Yuanye Bio-Technology Co., Ltd. Methanol (analytical reagent) was from National Drug Chemical Reagents Co., Ltd. Acetonitrile (chromatographic pure), formic acid (mass spectrometry reagent), and ammonium acetate (mass spectrometry reagent) were from Thermo Fisher Scientific. Water was of ultrahigh purity.

*2.2. Materials.* 25 batches of Long dan Xie gan Pill (LDXGW) samples were from 8 manufacturers (A-H, batch no. A01-A04, B01-B03, C01-C05, D01-D05, E01-E05, F01, G01, and H01).

*2.3. Instrumentation.* An Agilent 1260–6410B triple quadrupole LC-MS system (Agilent Technologies, Inc., Santa Clara, CA, USA) equipped with an electrospray ionization device was used for sample analysis. METTLER XS105 electronic analytical balance (Mettler-Toledo, Zurich, Switzerland), Milli-Q water purification system (Millipore, Burlington, USA), and KQ-300DA numerical control ultrasound cleaning instrument (Kun Shan Ultrasonic Instruments Co., Ltd., Kunshan, China) were used.

*2.4. Preparation of Standard Solutions.* Standard stock solutions of aristolochic acid I, aristolochic acid II, aristolochic acid IIIa, aristolochic acid IVa, 7-hydroxy aristolochic acid I, aristolactam I, aristolactam AIIIa, aristolactam BII, and aristolactam FI were prepared by dissolving suitable amounts of reference substance in methanol to make the concentration at 5 $\mu g \cdot mL^{-1}$, respectively.

*2.5. Preparation of Sample Solutions.* For Long dan Xie gan Pill (6 g per small bag), 5 bags were mixed and pulverized to powder. Then, 2 g was weighed accurately and put into a 50 mL plug conical bottle. Twenty-five mL methanol was added precisely and weighed, respectively. After extracting by ultrasonic extraction (power: 300 W; frequency: 40 kHz) for 30 min, the extract was cooled down and then made up for lost weight by adding methanol. The continuous filtrate was taken and then filtered by 0.22 $\mu$m microporous filter membrane.

*2.6. HPLC Chromatographic Condition.* Column: Agilent SB-C18 (2.1 × 50 mm, 1.8 $\mu$m); mobile phase: gradient elution with acetonitrile (A)-0.1% formic acid solution (containing 5 $mmol \cdot L^{-1}$ ammonium acetate) (B) (0–15 min, 31% A; 15–18 min, 31% A-60%A; 18–20 min, 60%A; 20–22 min, 60%A-31%A); flow rate: 0.3 $mL \cdot min^{-1}$; column temperature: 30°C; injection volume: 2 $\mu$L.

*2.7. MS Condition.* The triple quadrupole MS equipped with a positive electrospray ionization source was used in the MRM mode [22]. The equipment was set with a drying gas flow, nebulizer pressure, gas temperature, and spray voltage of 9 L·min, 30 psi, 350°C, and 4000 V, respectively.

The MRM conditions were individually optimized for each of the nine aristolochic acid analogues reference standards (AA I, AA II, AA IIIa, AA IVa, 7-OH AAI, AL I, AL AIIIa, AL BII, and AL FI) on account of their different structures [22]. The MS conditions for MRM are summarized in Table 1, and the typical MRM chromatogram is shown in Figure 2.

## 3. Result

The established LC-MS/MS method for 9 aristolochic acid analogues was applied to Long dan Xie gan Pills, and two aristolochic acids (AA I and AA IVa) and one aristolactam (AL I) were determined (Figure 3).

*3.1. Linearity.* Working standard solutions containing AA IVa, AL I, and AA I were prepared by diluting the stock mixed solution with methanol to a series of proper

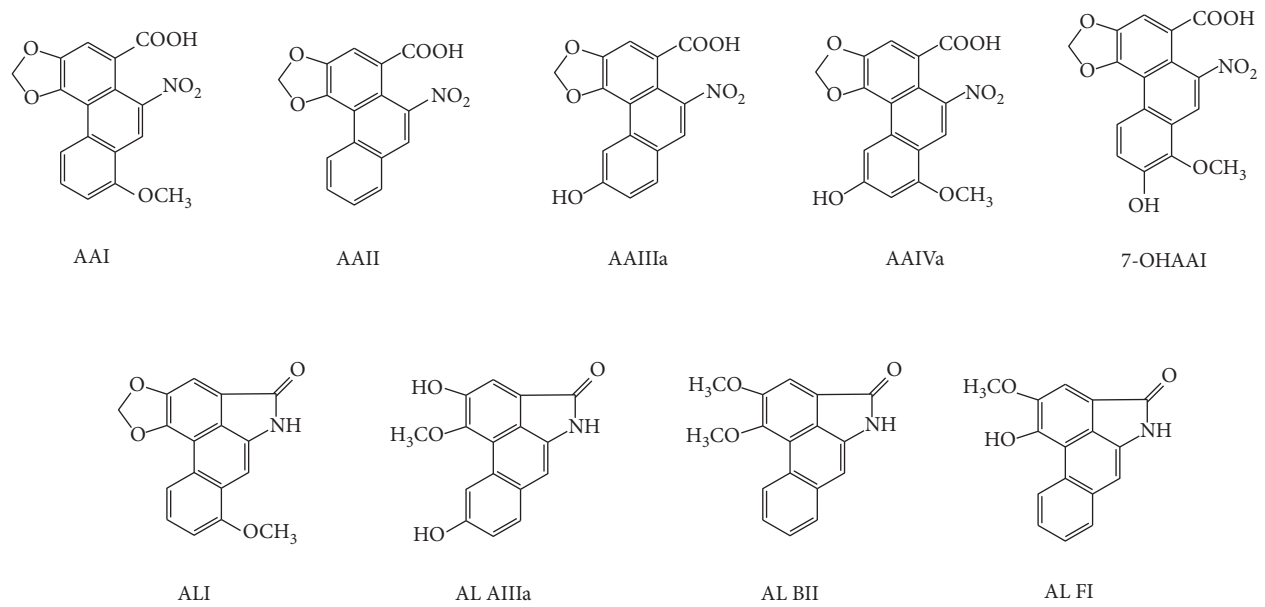


Figure 1: Chemical structures of aristolochic acid analogues.

Table 1: MS parameters for 9 AA analogues reference standards.

| No. | Compound | Retention time (min) | Precursor ion (m/z) | Production (m/z) | Fragmentor (V) | CE (eV) |
|---|---|---|---|---|---|---|
| 1 | AL AIIIa | 1.37 | 282.0 | 265.0* | 80 | 10 |
| | | | | 250.0 | 80 | 20 |
| 2 | AA IIIa | 2.32 | 345.0 | 282.0* | 60 | 8 |
| | | | | 284.0 | 60 | 5 |
| 3 | 7-OH AAI | 2.94 | 375.0 | 314.0* | 65 | 8 |
| | | | | 340 | 65 | 10 |
| 4 | AA IVa | 3.12 | 375.0 | 312.0* | 65 | 8 |
| | | | | 314.0 | 65 | 8 |
| 5 | AL FI | 3.93 | 266.0 | 251.0* | 125 | 25 |
| | | | | 195 | 125 | 30 |
| 6 | AA II | 9.21 | 329.0 | 268.0* | 70 | 8 |
| | | | | 294.0 | 70 | 5 |
| 7 | AL BII | 9.85 | 280.0 | 264.0* | 100 | 30 |
| | | | | 236.0 | 100 | 30 |
| 8 | AL I | 10.43 | 294.0 | 279.0* | 120 | 30 |
| | | | | 251.0 | 120 | 40 |
| 9 | AA I | 13.08 | 359.0 | 298.0* | 65 | 5 |
| | | | | 296.0 | 65 | 5 |

*The quantitative ions.

concentrations. Then, they were injected and analyzed. The results of regression equations, linearity, determination coefficient, and limits of detection and quantification of the method are presented in Table 2. All analytes presented a determination coefficient ($R^2$) of the 0.999 which allows the method to be considered linear.

*3.2. Limit of Detection and Limit of Quantification.* Precisely dilute the stock mixed solution with methanol quantitatively and stepwise if necessary. The diluted solutions were separately injected and analyzed. The limits of detection (LOD) and quantification (LOQ) (Table 3) were defined as the concentrations that could be detected and yield signal-to-noise (S/N) ratios of 3 : 1 and 10 : 1, respectively, according to guidelines for validation of analytical methods for pharmaceutical quality standards [23].

*3.3. Instrument Precision.* The same sample solution (A04) was injected for six consecutive times and analyzed. The RSDs of peak areas for AA IVa, AL I, and AA I were 5.13%, 4.62%, and 1.80%, respectively. It indicated the precision of the instrument was in accordance with requirement in guidelines for validation of analytical methods for pharmaceutical quality standards [23].

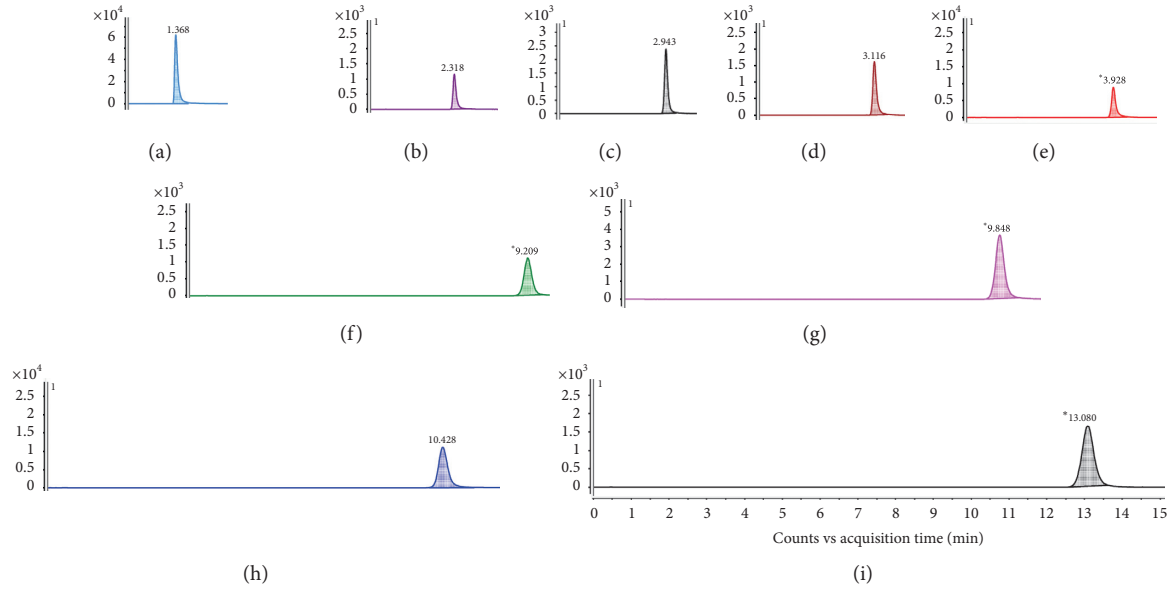


Figure 2: Typical MRM chromatograms of 9 aristolochic acid analogues reference standards. (a) AL AIIIa, (b) AA IIIa, (c) 7-OH AAI, (d) AA IVa, (e) AL FI, (f) AA II, (g) AL BII, (h) AL I, and (i) AA I.

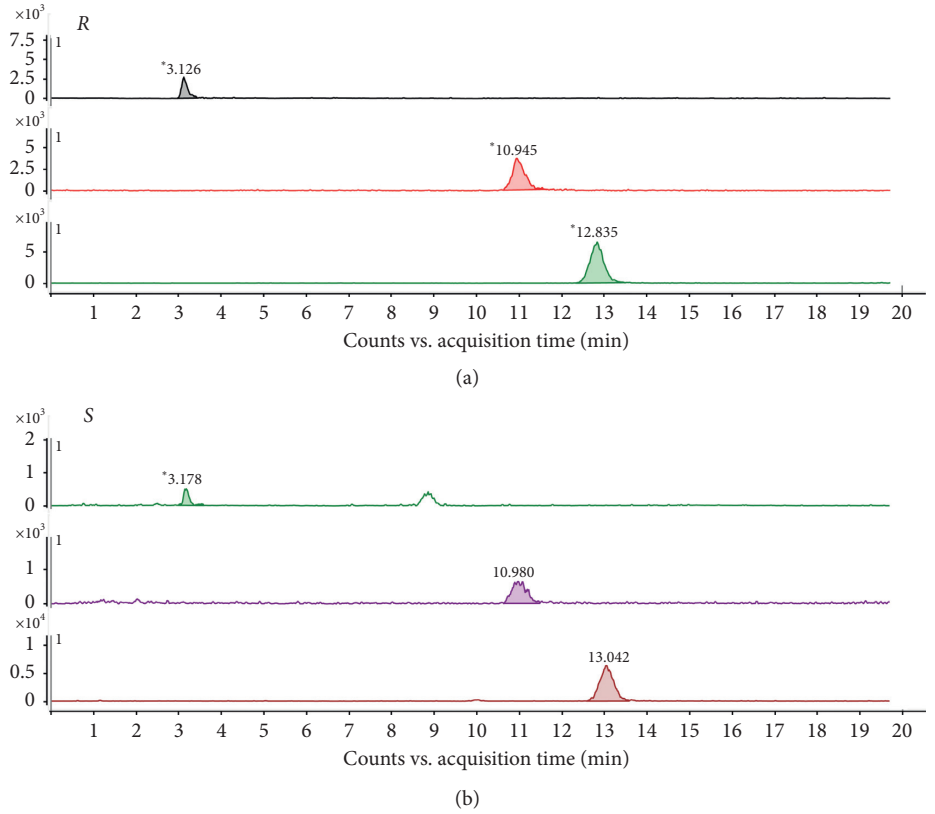


Figure 3: MRM chromatograms of reference standards and LDXGW sample (*R*: reference standards; *S*: sample A04).

*3.4. Repeatability.* The same batch of sample (A04) was taken and prepared for six independent sample solutions. Then, they were analyzed according to conditions under 2.6 and 2.7. The average contents of AA IVa, AL I, and AA I were 0.27, 0.27, 2.92 mg $\cdot g^{-1}$, respectively, and the RSDs were 3.75%, 6.16%, and 5.38%, respectively. It indicated method

Table 2: Regression equations, linear ranges, and coefficients for AA IVa, AL I, and AA I.

| Components | Regression equations | Linear ranges (pg) | $R^2$ |
|---|---|---|---|
| AA IVa | $y = 2.3306x - 1.2291$ | 9.702~116.424 | 0.9992 |
| AL I | $y = 9.0052x - 21.389$ | 13.524~162.288 | 0.9996 |
| AA I | $y = 4.6873 - 64.752$ | 96.6225~1159.47 | 0.9992 |

Table 3: LODs and LOQs for 9 aristolochic acid analogues.

| Components | AL AIIIa | AA IIIa | 7-OH AAI | AA IVa | AL FI | AA II | AL BII | AL I | AA I |
|---|---|---|---|---|---|---|---|---|---|
| LODs (pg) | 0.25 | 7.80 | 1.44 | 4.62 | 1.62 | 8.75 | 4.95 | 5.52 | 8.12 |
| LOQs (pg) | 0.74 | 23.40 | 4.32 | 13.85 | 4.85 | 26.25 | 14.85 | 16.56 | 24.38 |

repeatability was in accordance with requirement in guidelines for validation of analytical methods for pharmaceutical quality standards [23].

*3.5. Stability.* The same sample solution (A04) was injected at 0, 4, 8, 12, and 20 h at room temperature. The RSDs of peak areas for AA IVa, AL I, and AA I were 5.13%, 6.34%, and 4.74%, respectively. It indicated the sample solution was stable within 20 h.

*3.6. Recovery.* The recovery experiment was performed by adding a known amount of individual reference standards into a certain amount of sample (A04). Six separate samples of 1 g (contents of AA IVa, AL I, and AA I were 0.27, 0.27, 2.92 $mg \cdot g^{-1}$, respectively) were weighed accurately, and 1 mL of mixed reference standard solution (concentrations of AA IVa, AL I, and AA I were 0.5292, 0. 6811, 5.3068 $\mu g \cdot mL^{-1}$, respectively) was added then prepared samples according to 2.5. The results (Table 4) showed that the average recoveries ranged from 117.66% to 124.22% with RSDs in the range of 5.25%–6.04%, which indicated that the method was basically accurate.

*3.7. Sample Analysis.* Twenty-five batches of samples were prepared and analyzed according to 2.5, 2.6, and 2.7. The results displayed that sample A04 contained AA IVa, AL I, and AA I at the concentrations of 0.28, 0.25, and 2.67 $\mu g \cdot g^{-1}$, respectively. No aristolochic acids and aristolactams were detected in the other 24 samples.

## 4. Discussion

*4.1. Optimization of HPLC Chromatographic Conditions.* In our study, different mobile phase systems including acetonitrile-water, acetonitrile-0.1% formic acid, and acetonitrile-0.1% formic acid (containing 5 mM ammonium acetate) were investigated. As a result, the ionization intensities of aristolochic acids were best with ammonium acetate added in the mobile phase. Thus, the mobile phase including ammonium acetate was adopted.

*4.2. Optimization of MS Conditions.* During optimization of MS conditions for nine aristolochic acid analogues, it was shown that the precursor ions for the five aristolochic acids were all in the form of $[M + NH_4]^+$, while those for the four aristolactams were all in the form of $[M + H]^+$. This could be caused by the structural difference between aristolochic acids and aristolactams, and the main difference was that the existence form of nitrogen was nitro group and secondary amino group, respectively.

Further analysis indicated that both the product ions for quantitative and qualitative purpose of the five aristolochic acids were the fragmentation ions corresponding to $[M + H\text{-}NO_2]^+$, $[M + H\text{-}CO_2]^{+,}$ and $[M + H\text{-}H_2O]^+$. Although AA IVa and 7-OH AA I were isomers, their product ions were different due to the different substitution of hydroxyl and methoxyl groups. Also, the fragment ion at m/z 314 was to quantity for 7-OH AA I, but it was used to qualify for AA IVa. It should be mentioned that they could not be extracted at the same injection.

*4.3. Result Discussion.* Since there was no aristolochic acids contained in Akebiae Caulis (Mutong) in Long dan Xie gan Pill prescription, aristolochic acid I should not be detected. The determination results indicated that there was no substitution problem. It was possible that Akebiae Caulis (Mutong) was mixed with Caulis Aristolochiae manshuriensis (Guanmutong). Therefore, we should strictly control the quality of raw medicinal materials. In view of the harm of aristolochic acid I to human, the method has been applied for supplementary inspection method, and it provided sound scientific basis for further risk control measures and safeguard for drug safety.

*4.4. Determination of Aristolochic Acid Analogues.* Until now, there are more than 80 AAs and ALs found from the Aristolochiaceae family [24], and it would be much better if all these constituents could be determined. However, there are many limitations to accomplish this work. For quantitative analysis, reference substance is necessary to ensure the result accuracy. So far, there are only a few AAs and ALs reference substances. Furthermore, more than 80 AAs and ALs were mainly from the related crude drugs. Their contents were quite different, and many of them may exist in trace amounts. For traditional Chinese patent medicines that may contain AAs and ALs, the related crude drugs containing AAs and ALs were in low proportion of prescriptions. Based on our previous study and the literature research, the AAs and ALs with comparatively higher

Table 4: Recovery results of AA IVa, AL I, and AA I in Long dan Xie gan Pill samples.

| Components | No. | Sampling amount (g) | Sample content ($\mu$g) | Added amount ($\mu$g) | Detected amount ($\mu$g) | Recovery (%) | Average recovery (%) |
|---|---|---|---|---|---|---|---|
| AA IVa | 1 | 1.1835 | 0.3195 | 0.5292 | 0.9575 | 120.55 | 124.22% (RSD 5.25%) |
| | 2 | 1.1586 | 0.3128 | | 0.9311 | 116.84 | |
| | 3 | 1.1270 | 0.3050 | | 0.9509 | 122.04 | |
| | 4 | 1.0938 | 0.2953 | | 0.9452 | 122.81 | |
| | 5 | 1.1324 | 0.3057 | | 1.0226 | 135.47 | |
| | 6 | 1.1981 | 0.3235 | | 0.9988 | 127.62 | |
| AL I | 1 | 1.1835 | 0.3195 | 0.6811 | 1.1064 | 115.52 | 120.85% (RSD 6.04%) |
| | 2 | 1.1586 | 0.3128 | | 1.2322 | 134.98 | |
| | 3 | 1.1270 | 0.3050 | | 1.0925 | 115.62 | |
| | 4 | 1.0938 | 0.2953 | | 1.1221 | 121.38 | |
| | 5 | 1.1324 | 0.3057 | | 1.1083 | 117.83 | |
| | 6 | 1.1981 | 0.3235 | | 1.1391 | 119.75 | |
| AA I | 1 | 1.1835 | 3.4558 | 5.3068 | 9.0727 | 105.84 | 117.66% (RSD 5.39%) |
| | 2 | 1.1586 | 3.3831 | | 9.6685 | 118.44 | |
| | 3 | 1.1270 | 3.2987 | | 9.7298 | 121.19 | |
| | 4 | 1.0938 | 3.1939 | | 9.7422 | 123.39 | |
| | 5 | 1.1324 | 3.3066 | | 9.7375 | 121.18 | |
| | 6 | 1.1981 | 3.4985 | | 9.6487 | 115.89 | |

contents in crude drugs were studied in this manuscript. Considering the accuracy of detection results and research-related species, the LC-MS/MS determination method based on MRM mode for AL AIIIa, AA IIIa, 7-OH AAI, AA IVa, AL FI, AA II, AL BII, AL I, and AA I was established.

## 5. Conclusions

In this study, an efficient LC-MS/MS method based on MRM for 9 aristolochic acid analogues was established. It was also successfully applied for the commonly-used traditional Chinese patent medicine, Long dan Xie gan Pill. Since MRM has the outstanding advantages of strong specificity, high sensitivity, high accuracy, good reproducibility, and high throughput automation, the established method was demonstrated to be powerful for determination of the trace aristolochic acid analogues in complex traditional Chinese medicine. It also could provide scientific basis for the follow-up safety risk control measures.

## Data Availability

The electronic file of data used to support the findings of this study are available from the corresponding author upon request.

## Conflicts of Interest

The authors declare that there are no conflicts of Interest regarding the publication of this paper.

## Authors' Contributions

Jing Liu and Yang Liu contributed equally to this manuscript.

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