Pharmacogn. Mag.
A multifaceted peer reviewed journal in the field of Pharmacognosy and Natural Products
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ORIGINAL ARTICLE

Ultra-High Performance Liquid Chromatography/Triple Quadrupole Mass Spectrometry Study of the Stabilities and Transformations of Four Alisols in Alismatis Rhizoma and Proprietary Traditional Chinese Medicine Prescriptions

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Submitted: 28-12-2017 Revised: 16-02-2018 Published: 21-11-2018

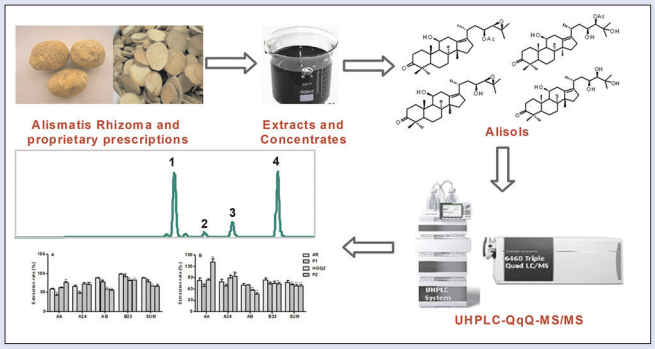
ABSTRACT

Background: Alismatis Rhizoma (AR) is a traditional Chinese medicine that is frequently used for the treatment of hyperlipidemia, hypertension, diabetes, and chronic kidney failure. Active alisols contribute to the beneficial effects of AR and AR products. **Objective:** In the present study, we investigated the stabilities and transformations of alisol B 23-acetate (B23), alisol A 24-acetate (A24), alisol A (AA), and alisol B (AB) in AR and proprietary traditional Chinese medicine prescriptions. **Materials and Methods:** We first established an ultra-high performance liquid chromatography/triple quadrupole mass spectrometry method for the analysis of AR products. Next, extracts and concentrates of AR and the proprietary prescriptions were prepared and heated at different temperatures. Finally, the developed method was applied to the analysis of these samples. **Results:** Our method is sensitive, precise, accurate, and reliable for the analysis of these four compounds. The extraction efficacies of the alisols from decoctions prepared from single herb and from mixtures of herbs showed large differences ($P < 0.01$). The alisols are highly sensitive to changes in temperature and pH and transform during heating. B23 can be transformed to AA and A24, and AB can be transformed to AA. **Conclusion:** These results indicate that the content change of aliols may affect the quality and efficacy of AR and AR products. Therefore, temperature and pH should be controlled during the manufacture of AR and AR products.

Key words: Alismatis rhizome, alisols, traditional Chinese medicine prescriptions, ultra-high performance liquid chromatography/triple quadrupole mass spectrometry

SUMMARY

- A simple, specific, and rapid ultra-high performance liquid chromatography/triple quadrupole mass spectrometry method for simultaneous quantification of four aliols in Alismatis Rhizoma and proprietary traditional Chinese medicine prescriptions has been developed
- The extraction efficacies of the alisols from decoctions prepared from single herb and from mixtures of herbs showed large differences ($P < 0.01$)
- The stabilities of the alisols are affected by the temperature (60°C–100°C) and pH (4.0–5.8).



Abbreviations used: AR: Alismatis Rhizoma; P1: Prescription 1; HGQZ: Hugan Qingzhi formula; P2: Prescription 2; AA: Alisol A; A24: Alisol A 24-acetate; AB: Alisol B; B23: Alisol B 23-acetate; UHPLC–QqQ–MS/MS: Ultra-high performance liquid chromatography/triple quadrupole mass spectrometry.

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DOI: 10.4103/pm.pm_614_17

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INTRODUCTION

Alismatis Rhizoma (AR), the dried rhizomes of *Alisma orientalis* (Sam.) Juzep. (family *Alismataceae*), is a traditional Chinese medicine (TCM) frequently used for the treatment of hyperlipidemia, hypertension, diabetes, and chronic kidney failure and as a diuretic.[1-5] The major chemical components of AR are protostane-type triterpenes, among which alisol B 23-acetate (B23) and alisol A 24-acetate (A24) are the major active compounds responsible for lowering lipid levels. Pharmacological

Cite this article as: Xiao CX, He F, Yang MT, Xia F, Tang WJ, Zhou BJ, *et al.* Ultra-high performance liquid chromatography/triple quadrupole mass spectrometry study of the stabilities and transformations of four alisols in Alismatis Rhizoma and proprietary traditional Chinese medicine prescriptions. Phcog Mag 2018;14:597-603.

studies have indicated that B23 is potentially useful for the treatment of metabolic diseases, such as nonalcoholic steatohepatitis.[6,7] A24 can scavenge accumulated triglycerides and cholesterol *in vivo* and *in vitro*.[8,9] Alisol A (AA) and alisol B (AB) are the other major compounds responsible for the pharmacological activity of AR.[10] Because of their relatively high contents and proven contributions to the bioactivity of AR, these four alisols as shown in Figure 1 are commonly used as quality markers of AR and proprietary TCM preparations.

Previous studies have revealed that alisols in AR are unstable and their contents change after traditional processes such as drying, salting, and brining.[11] In addition, B23 and A24 are unstable under simulated stomach conditions (pH 1.7–3.0), with B23 transformed rapidly to A24, potentially followed by further transformation to AA and an unknown compound.[12] These studies focused on the content change of aliols during superheating or digestion; however, the stability of aliols in the process of ordinary manufacture was not be taken into consideration. Besides, the transformation of alisols in AR was simply studied using thin-layer chromatography or liquid chromatography coupled with ultraviolet detector (LC–UV). The general procedure for the preparation of AR involves boiling it in water or ethanol at 70°C–100°C, and it is unclear whether alisols are stable during this process. Moreover, in medicinal preparations, AR is likely to be affected by acidic media during mixing of decoctions and further preparation. To ensure the quality, safety, and efficacy of AR, the factors that influence the stabilities of bioactive alisols should be controlled in the preparation of AR and AR proprietary TCM preparations. To date, chemical profiling and analysis of multiple compounds in AR have been performed by LC–UV,[13] LC coupled with evaporative light scattering detector (LC–ELSD),[14] and ultra-high performance liquid chromatography/quadrupole time-of-flight mass spectrometry (UHPLC/Q–TOF–MS).[15] However, few studies reported the qualitative and quantitative analyses of alisols in AR proprietary TCM preparations. UHPLC-triple quadrupole MS (UHPLC–QqQ–MS) is an effective method with high sensitivity and is frequently applied to the analysis of TCMs. Therefore, a UHPLC–QqQ–MS/MS method can be established for simultaneous quantitative analysis of four alisols in AR and AR proprietary TCM preparations.

The aim of the present study was to develop a rapid and sensitive UHPLC–QqQ–MS/MS method for simultaneous quantitative analysis of four alisols in AR products. Besides, we carefully investigated the effects of temperature and pH on alisols in AR and an AR proprietary TCM during the processes of traditional decoction and concentration. We used the following AR proprietary TCM: Hugan Qingzhi (HGQZ), prescription 1 (P1), and prescription 2 (P2). HGQZ was formulated with AR, Crataegi Fructus (CF), Nelumbinis Folium (NF), Typhae Pollen (TP), and Notoginseng Radix (NR). P1 was prepared from AR, NF, TP, and NR, and P2 was prepared from AR and CF. We supposed that transformation of alisols in AR, HGQZ, P1, and P2 may take place during heating for extraction and concentration. Therefore, extracts and concentrates were obtained from AR, HGQZ, and two prescriptions. The extraction efficacies of the alisols were compared for the various extracts and concentrates. To evaluate the influence of pH on the alisols, the pH values of the extracts and concentrates were determined. The influence of temperature on the alisols was studied by dividing the extracts and concentrates into several groups that were heated at different temperatures. Finally, the UHPLC–QqQ–MS/MS method was applied to determine the contents of the alisols in each sample.

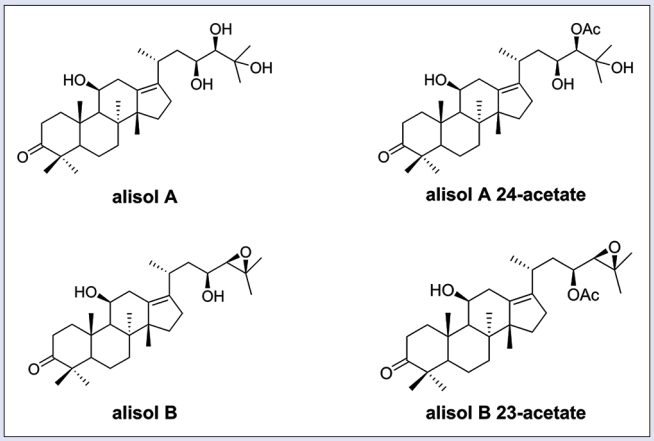

Figure 1: Chemical structures of the alisols (78.8 mm × 120 mm)

MATERIALS AND METHODS

Herbal materials, chemicals, and reagents

AR (Batch No.: 20160801), FC (Batch No.: 161208), PN (Batch No.: 161104), PT (Batch No.: 160701), and RN (Batch No.: 161212) were collected from Good Agricultural Practice of Medicinal Plants and Animals bases in Fujian, Shandong, Shandong, Anhui, and Yunnan Provinces, respectively. These materials were authenticated in line with the Chinese Pharmacopoeia[16] by Chunsong Chen (State Key Laboratory of Quality Research in Chinese Medicine, Macau University of Science and Technology), an expert in herbal authentication, and voucher specimens were deposited at the State Key Laboratory.

Standard reference compounds (purity ≥98%) of AA, A24, and AB were purchased from Beijing Century Aobo Biotechnology Co., Ltd. [Beijing, China]. A standard of B23 [purity ≥98%]) was purchased from the National Institutes for Food and Drug Control (Beijing, China). HPLC-grade acetonitrile was obtained from Anaqua Chemicals Supply (Houston, TX, USA). Distilled water was further purified using a Milli-Q system (Millipore, Milford, MA, USA). All other chemicals were of analytical grade. All solvents and samples were filtered through 0.22-μm filters before injection into the UHPLC.

Instrumentation and ultra-high performance liquid chromatography-mass spectrometry conditions

A UHPLC system (1290 series, Agilent Technologies, Santa Clara, CA) coupled with an Agilent 6460 Triple Quadrupole Mass Spectrometer (QqQ–MS, Agilent Technologies) was used to quantitatively detect the four alisols. Chromatographic separation was performed on a Waters Acquity UPLC C18 (2.1 mm × 100 mm, 1.7 μm; Waters, Milford, MA, USA) at 30°C. The mobile phase consisted of 0.1% formic acid and acetonitrile with a gradient elution of 50%–90% acetonitrile from 0 to 5 min. The sample injection volume was 2 μL, and the flow rate was 0.35 mL/min. Detection of AA and A24 was performed using multiple reaction monitoring (MRM), with an electrospray ionization source in negative ion mode, and that for AB and B23 was performed in positive ion mode. The transitions of the four compounds were m/z 489.5 → 471.3 (Frag 240, CE10) for AA, m/z 577.4 → 531.4 (Frag 140, CE 10) for A24, m/z 495.3 → 381.1 (Frag 120, CE 14) for AB, and m/z 515.4 → 107.1 (Frag 140, CE 98) for B23. The other parameters were as follows: drying gas (N_2) flow rate, 11.0 L/min; drying gas temperature, 300°C; nebulizer, 15 psig; and capillary voltage, 4000 V.

Preparation of the Alismatis Rhizoma standard solution

According to the Chinese Pharmacopoeia, a sample of 0.5 g of AR was accurately weighed and then extracted for 20 min with 25 mL of acetonitrile in an ultrasonic water bath. All measurements were repeated in triplicate.

Preparation of extracts

Extracts were prepared as follows: AR from AR (36 g); HGQZ from AR (36 g), CF (36 g), NF (24 g), TP (18 g), and NR (9 g); P1 from AR (36 g), NF (24 g), TP (18 g), and NR (9 g); and P2 from AR (36 g) and CF (36 g). For each TCM, four separate samples were refluxed three times with six times the volume of 70% ethanol for 1 h and each time. The ethanol from each reflux was combined, and the final volume of the mixed ethanol extract was diluted to 2000 mL with 70% ethanol. All preparations were repeated in triplicate. 10 mL of each extract of AR, HGQZ, P1, and P2 was retained for measurement, and the residual extracts from triplicate preparations were used for concentration.

Preparation of concentrates

One thousand milliliters of each extract prepared from the section "Preparation of extracts" was concentrated separately to 200 mL at 60°C. 10 mL of each concentrates of AR, HGQZ, P1, and P2 was retained for measurement, and the residual extracts were mixed, respectively.

Preparation of heat-treated samples

The extract mixture and the concentrate mixture were each divided into 27 tubes with 15 mL in each tube. Each set of 27 tubes was divided into three groups, with nine tubes in each group. The tubes were heated at 60°C, 80°C, or 100°C in water bath for 1, 2, or 8 h.

Determination of the pH

The pH values of the extracts and concentrates were tested, with a pH indicator (Starter 2100/3CPro, Ohaus, USA). All measurements were repeated in triplicate.

Standard solutions and sample preparation

Stock solutions of each of the four reference standards were prepared in methanol and then stored at 4°C. A mixed standard solution was obtained by accurately mixing the four stock solutions and then diluting with methanol. The final concentrations of AA, A24, AB, and B23 in the mixture were 93.0, 124.0, 115.0, and 224.0 μg/mL, respectively. The concentrations of the standard solutions in Table 1 were obtained by diluting the mixed standard solution. An aliquot (2 μL) of the standard solution was injected into the UHPLC–QqQ–MS system, and the representative MRM chromatograms of the four reference standards were obtained [Figure 2].

The extract and concentrate were diluted with methanol in 2:5 and 2:25 volume ratios, respectively, and filtered through 0.22-μm syringe filters. The filtrates were used as test solutions and analyzed by UHPLC–QqQ–MS/MS, and the representative MRM chromatograms of AR, P1, HTQZ, and P2 were obtained [Figure 2].

Statistical analysis

Statistical analysis was performed with SPSS 20.0 software (International Business Machines, Armonk, NY, USA), and an analysis of variance was used to compare means in different groups followed by Bonferroni's multiple comparisons test.

RESULTS AND DISCUSSION

Optimization of ultra-high performance liquid chromatography/mass spectrometry conditions

To achieve the best possible resolution and symmetrically shaped peaks of the four compounds within a suitable runtime, different kinds of mobile phases such as acetonitrile and methanol with a variety of modifiers were optimized. It was found that the mobile phase with acetonitrile had better resolutions and shorter duration of analysis than

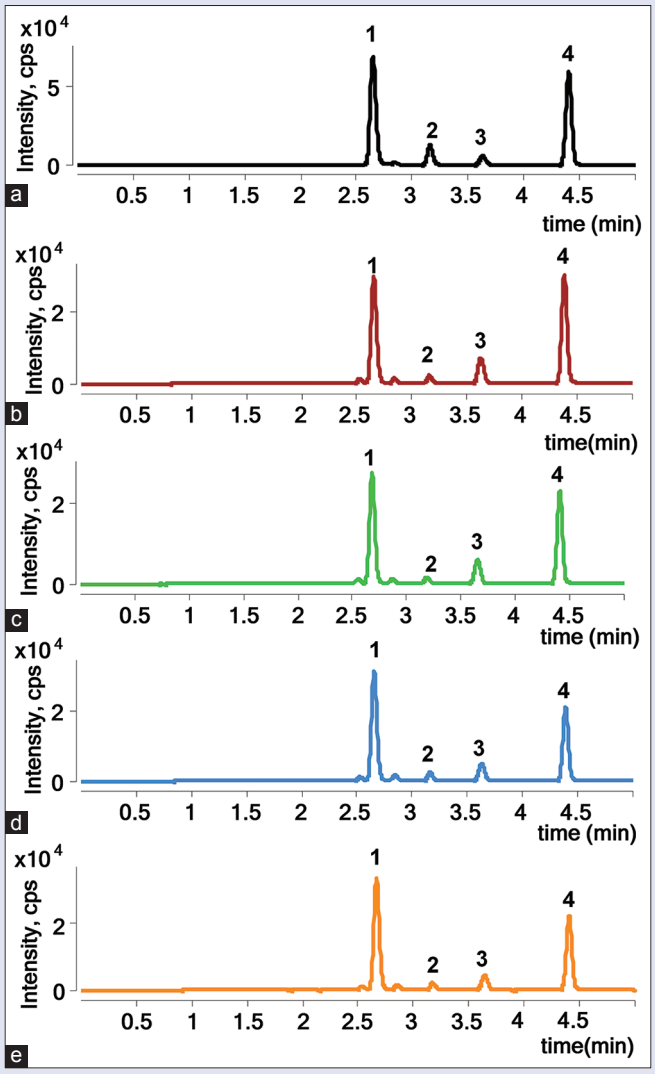

Figure 2: Representative multiple reaction monitoring chromatograms of (a) standard mixture, (b) Alismatis Rhizoma, (c) prescription 1, (d) Hugan Qingzhi, and (e) prescription 2. The peak labels are for alisol A (1), alisol A 24-acetate (2), alisol B (3), and alisol B 23-acetate (4) (80 mm × 50 mm)

Table 1: Calibration curves, ranges, and limits of determination and quantification

Analyte	Linearity			LOD (ng/mL)	LOQ (ng/mL)
	Calibration curves	R	Ranges (μg/mL)		
AA	y=184.68x + 80.6	0.9994	0.511~10.2	3.19	5.11
A24	y=342.68x + 5.9032	0.9993	0.0930~3.72	0.930	1.86
AB	y=2464.8x + 675.58	0.9991	0.201~8.05	5.03	10.1
B23	y=9429.1x + 828.5	0.9996	0.140~22.4	2.80	5.60

AA: alisol A; A24: alisol A 24-acetate; AB: alisol B; B23: alisol B 23-acetate

those with methanol. Formic acid at a low concentration (0.1%, v/v) was added to improve the ionization responses and to minimize peak tailing. The acetonitrile and water (0.1% formic acid) was eventually selected as the mobile phase. The signals of AA and A24 were detected in negative ion mode; abundant information of B and B23 was obtained in positive ion mode. Therefore, both the positive and negative ion modes were employed for comprehensive analysis. The collision energy and fragmentor voltage parameters were optimized to obtain the highest relative abundance of the exclusive ions and product ion in the MRM optimization conditions [Figures S1 and S2]. The final conditions for the collision energy and fragmentor voltage are shown in the section "Instruments and UHPLC-MS conditions."

Method validation

Linearity, range, and limits of determination and quantification

After testing the calibration standards, calibration curves were constructed by plotting the peak areas versus concentrations. The linearity parameters for the investigated compounds are listed in Table 1. All calibration curves showed good linearity ($r > 0.999$) within the tested ranges. The limits of detection (signal-to-noise ratio = 3) and the limits of quantification (signal-to-noise ratio = 10) for the four compounds were in the ranges of 0.930–5.03 ng/mL and 1.86–10.1 ng/mL, respectively.

Precision, repeatability, stability, and accuracy

The precision was examined by analyzing a mixed standard solution six times over 1 day. The repeatability was examined by analyzing six samples prepared by the same method. The stability was tested over 0, 2, 4, 6, 8, and 12 h in 1 day. The results showed that the assay was satisfactory with respect to precision, repeatability, and stability, demonstrated by all relative standard deviations (RSDs) <5% [Table 2]. The recovery was validated by adding a known volume of a stock standard solution to a certain amount of HGQZ. The mixtures were analyzed in triplicate with the optimized method. The recoveries were calculated using the formula: recovery (%) = (detected amount − original amount)/spiked amount × 100. The recovery of this method varied from 96.6% to 103% with an RSD between 2.30% and 3.44% [Table 2], showing a satisfactory accuracy.

Influence of the decoction preparation conditions on the alisols

In this experiment, AR, P1, HGQZ, and P2 were extracted separately with 70% ethanol three times, and the contents of the alisols in the extracts were determined by UHPLC–QqQ–MS/MS. In the extracts of AR, P1, HGQZ, and P2, the highest content was observed for B23, followed by AB, AA, and A24. The extraction efficacies of the alisols from decoctions prepared from single herb (AR) and from mixtures of herbs (P1, HGQZ, and P2) showed large differences [Figure 3a]. More B23 and AB were extracted from AR and P1 than from HGQZ and P2, whereas more AA and A24 were extracted from HGQZ and P2 than from AR and P1. These results revealed that high extraction rates were achieved for B23 and AB from AR on its own, but the extraction efficacy greatly decreased when AR was mixed with other TCMs, and especially with CF. By contrast, higher extraction rates were obtained for AA and A24 when AR was mixed with CF. The total extraction efficacies of the four alisols were 87.65%, 77.28%, 67.27%, and 66.30% for the extracts of AR, P1, HGQZ, and P2, respectively. The pH values decreased in the following order: AR > P1 > HGQZ > P2 [Figure 4A]. This indicated that the pH was closely related to the increases in the levels of AA and A24 in extracts from HGQZ and P2 during the extraction process.

Influence of the concentration conditions on the alisols

In this experiment, a concentrate was prepared from each extract at 60°C under reduced pressure, and the contents of the alisols in each concentrate were determined by UHPLC–QqQ–MS/MS. Compared with the extracts, the levels of B23 and AB decreased rapidly and the contents of A24 and AA increased dramatically in the concentrates of AR, P1, HGQZ, and P2 [Figure 3b]. The total extraction efficacies of the four alisols were 77.74%, 77.86%, 68.42%, and 68.52% in the concentrates from AR, P1, HGQZ, and P2, respectively. These results demonstrate that transformation of the alisols has taken place during the concentration.

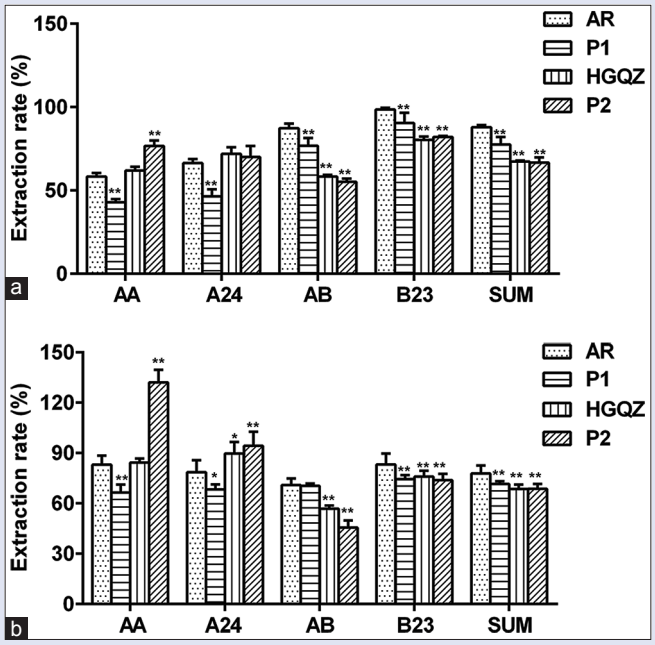

Figure 3: The extraction rates of alisol A (AA), alisol A 24-acetate (A24),alisol B (AB), alisol B 23-acetate (B23), and the sum of the four alisols (SUM) in the extracts (a) and concentrates (b) from Alismatis Rhizoma, prescription 1, Hugan Qingzhi formula, and prescription 2. **p < 0.01 vs. Alismatis Rhizoma group, *p < 0.05 vs. Alismatis Rhizoma group. (70 mm × 133 mm)

Table 2: Relative standard deviations (RSDs) for the precision, repeatability, stability, and recovery of each of the four alisols

Analyte	Precision RSD (%) (*n*=6)	Repeatability RSD (%) (*n*=6)	Stability RSD (%) (*n*=6)	Recovery (*n*=6)				
				Original (μg)	Spiked (μg)	Detected (μg)	Recovery (%)	RSD (%)
AA	2.53	3.40	2.92	4.53	4.46	8.99	99.7	3.44
A24	3.66	4.37	4.82	0.920	0.905	1.83	100	2.97
AB	1.05	2.23	1.15	1.62	1.61	3.17	96.6	2.30
B23	0.830	3.60	1.18	2.67	2.80	5.56	103	2.50

AA: alisol A; A24: alisol A 24-acetate; AB: alisol B; B23: alisol B 23-acetate

A previous study showed that the transformation of B23 can occur via two routes when AR is processed at a high temperature (160°C–200°C). In one pathway, B23 is rearranged into A24, which could be deacetylated into AA, and in the other pathway, it is deacetylated to AB before transformation into AA.[17,18] In the present study, B23 and AB were transformed into AA and A24 when heated at a relatively low temperature (60°C). Interestingly, transformations from B23 to AB and from A24 to AA were not observed in this study. Previous study has been reported that alisols are stable when the pH is above 4.0.[12] However, in our study, the pH values of the concentrates of AR, P1, and HGQZ were about 4.0–5.8 [Figure 4B]. In the concentrate of P2, the extraction rates of AA and A24 increased to 132% and 94%, respectively. This indicates that a small amount of acids accelerated the transformations from B23 and AB to A24 and AA. Therefore, it is important that manufacturers pay attention to the interactions among different components in proprietary TCM preparations with AR as one component.

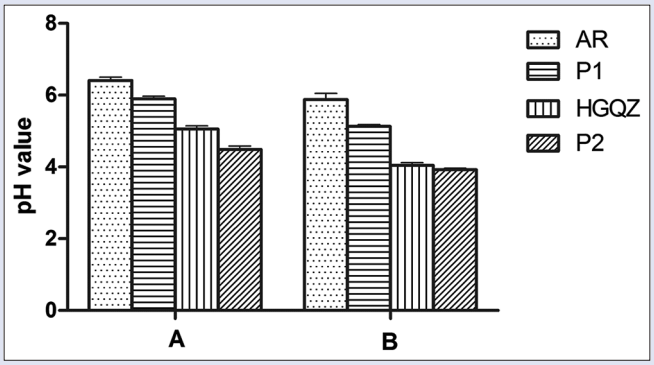

Figure 4: pH values of the extracts (A) and concentrates (B) from Alismatis Rhizoma, prescription 1, Hugan Qingzhi formula, and prescription 2. Values are expressed as mean ± standard deviation in each group (n = 3) (70 mm × 120 mm)

Influence of heat treatment on the alisols

In this experiment, the extracts and concentrates were divided into several groups and heated at 60°C, 80°C, or 100°C for 1 h, 2 h, or 8 h. Subsequently, the contents of the alisols in the samples were determined by UHPLC–QqQ–MS/MS.

Figures 5-8 show changes in the contents of AA, A24, AB, and B23 in the extracts and concentrates of AR, P1, HGQZ, and P2 during the

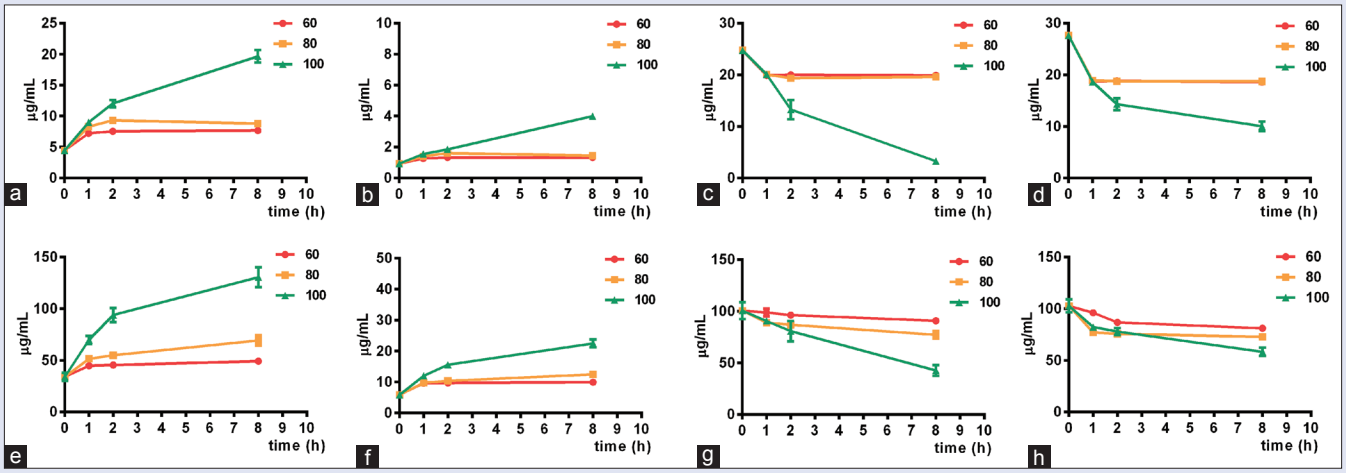

Figure 5: Influence of temperature on alisols in extracts (a-d) and concentrates (e-h) from Alismatis Rhizoma. (a and e) alisol A, (b and f) alisol A 24-acetate, (c and g) alisol B, and (d and h) alisol B 23-acetate (80 mm × 216 mm)

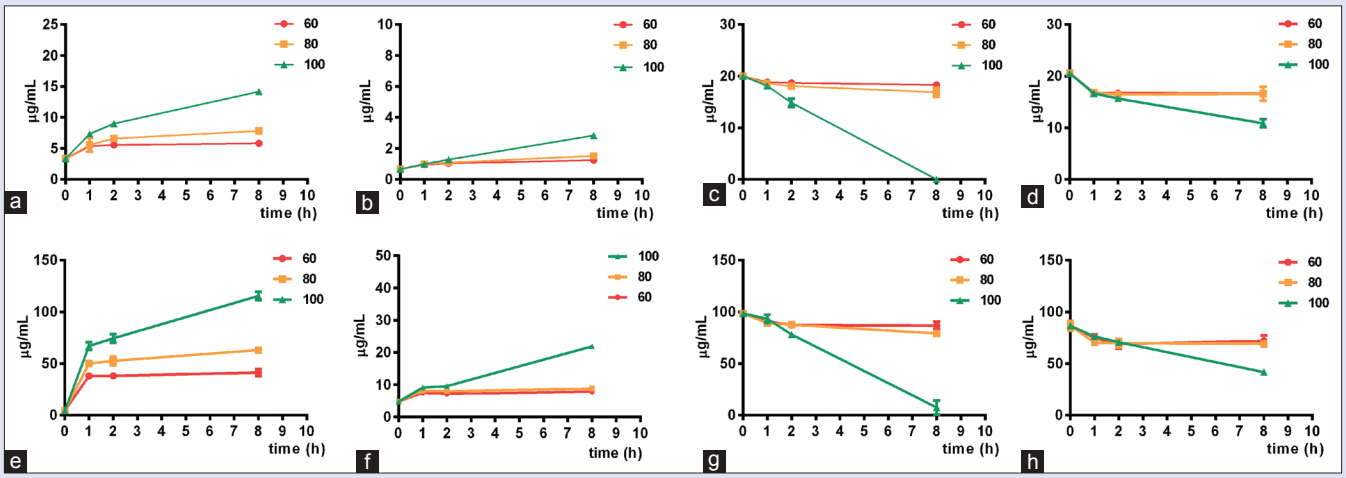

Figure 6: Influence of temperature on alisols in the extracts (a-d) and concentrates (e-h) from prescription 1. (a and e) alisol A, (b and f) alisol A 24-acetate, (c and g) alisol B, and (d and h) alisol B 23-acetate (80 mm × 216 mm)

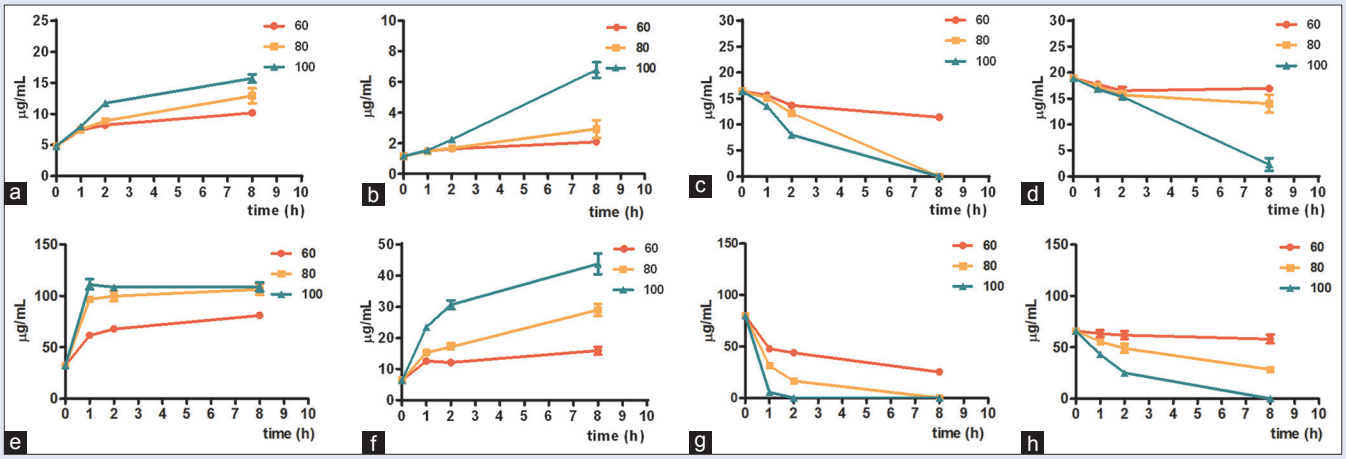

Figure 7: Influence of temperature on alisols in the extracts (a-d) and concentrates (e-h) from Hugan Qingzhi. (a and e) alisol A, (b and f) alisol A 24-acetate, (c and g) alisol B, and (d and h) alisol B 23-acetate (80 mm × 216 mm)

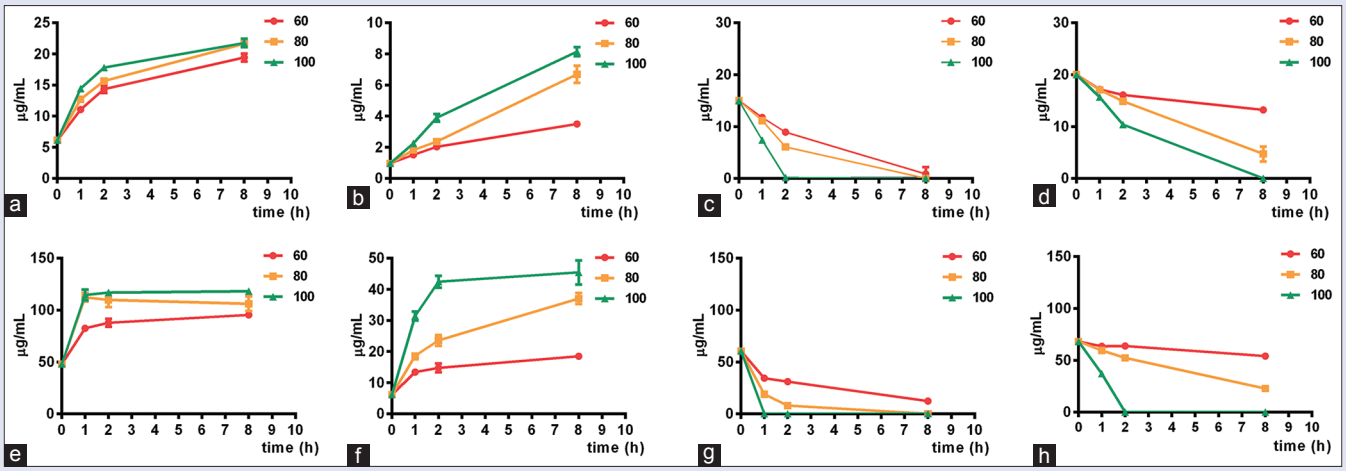

Figure 8: Influence of temperature on alisols in the extracts (a-d) and concentrates (e-h) from prescription 2. (a and e) alisol A, (b and f) alisol A 24-acetate, (c and g), alisol B, and (d and h) alisol B 23-acetate (80 mm × 216 mm)

heating process. For AR [Figure 5a-d], changes in the contents of the alisols at 60°C and 80°C were similar, with the levels of B23 and AB decreasing slightly and the levels of AA and A24 increasing within 2 h. After that time, the levels of the alisols were stable with continued heating. However, for samples heated at 100°C, the contents of B23 and AB decreased dramatically and those of AA and A24 increased rapidly within 8 h. After concentration [Figure 5e-g], these transformations were magnified. The results for P1 [Figure 6] were identical to those for AR. The only difference was that the level of AB decreased faster in the concentrate from P1 than in that from AR.

For the HGQZ samples [Figure 7], the contents of B23 and AB showed downward trends as the heating duration increased, and the final concentration of AB was extremely low when the sample was heated at 100°C for 8 h. By contrast, the contents of AA and A24 increased as the temperature and duration of heating increased. The trends observed for the P2 samples [Figure 8] were identical to those observed for the HGQZ samples. However, AB decomposed faster and was not detected in the concentrates of P2 after heating at 60°C or 80°C for 8 h and at 100°C for 2 h.

Our data demonstrate that B23 and AB are rapidly transformed within 2 h during heat treatment, followed by a gradual decrease in the transformation rate. Increased acidity further enhanced the instabilities of the alisols. Furthermore, AB was more sensitive to temperature and pH than B23. In addition, the transformations from B23 to AB and A24 to AA did not occur at high temperature and/or with high acidity.

Based on these results, we found that changes in both temperature and pH induced transformations of the alisols. We believe that B23 can transform to AA and A24, and AB can transform to AA at temperatures of 60°C–100°C, with a pH of 3.9–6.0 [Figure S3]. This transformation can be accelerated by increasing the temperature or acidity. Therefore, manufacture of B23 and AB should be conducted at low temperature and acidity. However, high temperature and acidity contributes to production of AA and A24, which are present in low levels in AR.

CONCLUSION

Our UHPLC–QqQ–MS/MS method provides rapid and sensitive detection of four alisols simultaneously in extracts from AR, P1, HGQZ, and P2. Both the sensitivity and accuracy of this method are superior to LC–UV in studying the stability and transformation of alisols in AR. The current research found that the stabilities of the alisols are affected by the temperature (60°C–100°C) and pH (4.0–5.8); these parameters should

be controlled in the preparation processes for AR and AR-containing products. Besides, the transformation pathway of alisols in decoction and concentration is not the same with the results from previous studies. Transformation from B23 and AB to A24 and AA reduces the contents of B23 and AB and increases the levels of AA and A24, which implies that the medicinal properties and clinical efficacies of AR and AR proprietary TCMs might be affected. Therefore, changes in the levels of the alisols during the manufacture of AR and AR-containing products should be monitored. Previous studies have verified the antihyperlipidemic and anti-atherosclerotic activities of B23 and A24.[7,19] However, few studies have reported the pharmacological activities of AA and AB. The existing information is insufficient to explain whether this transformation will positively or negatively affect the pharmacological efficacy of AR. Therefore, this should be investigated further in the future pharmacological studies.

Acknowledgments

C.X.X.and F.H.performed the LC-MS analyses. C.X.X., M.T.Y., F.X.and W.J.T. conducted the stability study. B.J.Z.and H.Z.designed the study. C.X.X., B.J.Z. and H.Z.analyzed the data and prepared the manuscript.

Financial support and sponsorship

This research was supported by Science and Technology Project of Guangzhou (No. 201604020033) and Scientific and Technological Development and Cultivation Project of Southern Medical university (No.KJ20160503).

Conflicts of interest

There are no conflicts of interest.

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