Development and validation of an high-performance liquid chromatography-diode array detector method for the simultaneous determination of six phenolic compounds in abnormal savda munziq decoction

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ABSTRACT

Aims: Given the high-effectiveness and low-toxicity of abnormal savda munziq (ASMQ), its herbal formulation has long been used in traditional Uyghur medicine to treat complex diseases, such as cancer, diabetes, and cardiovascular diseases. **Settings and Design:** ASMQ decoction by reversed-phase high-performance liquid chromatography coupled with a diode array detector was successfully developed for the simultaneous quality assessment of gallic acid, protocatechuic acid, caffeic acid, rutin, rosmarinic acid, and luteolin. The six phenolic compounds were separated on an Agilent TC-C18 reversed-phase analytical column (4.6 × 250 mm, 5 μm) by gradient elution using 0.3% aqueous formic acid (v/v) and 0.3% methanol formic acid (v/v) at 1.0 mL/min. **Materials and Methods:** The plant material was separately ground and mixed at the following ratios (10): *Cordia dichotoma* (10.6), *Anchusa italic* (10.6), *Euphorbia humifusa* (4.9), *Adiantum capillus-veneris* (4.9), *Ziziphus jujube* (4.9), *Glycyrrhiza uralensis* (7.1), *Foeniculum vulgare* (4.9), *Lavandula angustifolia* (4.9), *Dracocephalum moldavica* L. (4.9), and *Alhagi pseudoalhagi* (42.3). **Statistical Analysis Used:** The precisions of all six compounds were <0.60%, and the average recoveries ranged from 99.39% to 104.85%. Highly significant linear correlations were found between component concentrations and specific chromatographic peak areas (R^2 > 0.999). **Results:** The proposed method was successfully applied to determine the levels of six active components in ASMQ. **Conclusions:** Given the simplicity, precision, specificity, and sensitivity of the method, it can be utilized as a quality control approach to simultaneously determining the six phenolic compounds in AMSQ.

Key words: Abnormal savda munziq decoction, diode array detector, high-performance liquid chromatography, phenolic compounds, uighur medicine

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INTRODUCTION

The herbal formulation abnormal savda munziq (ASMQ) tang of the traditional Uighur medicine from the Xinjiang region of China is used to treat complex diseases such as cancer, diabetes, and cardiovascular diseases.[1-5] This formulation is composed of 10 medicinal herbs, namely, *Cordia dichotoma* Forst. f, *Anchusa italica* Retz., *Euphorbia humifusa* Willd., *Adiantum capillus-veneris* L., *Ziziphus jujube* Mill., *Glycyrrhiza uralensis*, *Foeniculum vulgare* Mill., *Lavandula angustifolia* Mill., *Dracocephalum moldavica* L., and *Alhagi pseudoalhagi* Desv.[6] Traditional Uighur medicine is an important component of traditional Chinese medicine that has its own traditional theories for preventing and treating cancer. Traditional Uighur medicine has been used for pharmaceutical and dietary purposes for several millennia.

Abnormal savda munziq can reportedly scavenge free radicals,[7] protect mitochondria and deoxyribonucleic acid (DNA) from oxidative damage,[8] as well as significantly inhibit the growth and viability of the human hepatoma cell line HepG2 by increasing cytopl asmic leakage and inhibiting protein, DNA, and RNA synthesis.[4] ASMQ also shows protective and reparative effects in Abnormal Savda

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carrier animal models as revealed by organ histopathology studies.[9] The phenolic compounds have indeed been proven to play a vital role in the protection against cancer and may thus also affect the immune system.

The constituents of ASMQ are very complex, and traditional Chinese medical prescriptions are rich in carbohydrates. ASMQ and the carbohydrates present in Chinese medical prescriptions are difficult to separate. Several papers have reported the discovery of all major active components in ASMQ, such as flavonoids, phenolics, saccharides, and saponins. However, preliminary studies have only explored the phenolic chemical composition of ASMQ. Single polyphenol or flavonoids have been examined by only a few studies and are thus unclear.[10] Accordingly, the present study aimed to establish a simple, efficient, and sensitive method for simultaneously analyzing six phenolic compounds: luteolin, gallic acid, caffeic acid, rutin, rosmarinic acid,[11] and protocatechuic acid [Figure 1] in ASMQ for quality control based on high-performance liquid chromatography (HPLC) coupled with a diode array detector (DAD).[12,13] All samples were extracted and analyzed in triplicate.

MATERIALS AND METHODS

Materials and reagents

Medicinal plants, *C. dichotoma, A. italica, E. humifusa, Z. jujube, G. uralensis, F. vulgare, L. angustifolia, D. moldavica* and *A. pseudoalhagi* were purchased from Xinjiang Maidisen Uyghur Medicine Co., Ltd (Xinjiang, China). The plant materials identified by Yonghe Li, a chief apothecary of the Chinese Medicine Hospital of Xinjiang. The Standard of protocatechuic acid was purchased from the Shanghai Institute of Biological Products Co., Ltd (Shanghai, China). The analytical grade standard of luteolin and gallic acid were purchased from Mansite companies (Chengdu, China). The analytical grade standard of caffeic acid and rutin were purchased from the National Institute for the control of Pharmaceutical and Biological Products (Beijing, China). The analytical grade standard of rosmarinic acid was obtained from Sigma-Aldrich (USA). Analytical grade formic acid was obtained from Tianjin, Fuyu Chemical Reagent Company. HPLC-grade methanol was supplied by Fisher Scientific (USA), and water was obtained from Millipore Q3 ultra-pure water system (Millipore, USA).

Preparation of abnormal savda munziq and standard-solutions

Ten medical plants were pulverized into a fine powder with particle sizes of 40-60 mesh using a stainless steel blender. The plant material was separately ground and mixed at the following ratios (10): *C. dichotoma* (10.6), *A. italic* (10.6), *E. humifusa* (4.9), *A. capillus-veneris* (4.9), *Z. jujube* (4.9), *G. uralensis* (7.1), *F. vulgare* (4.9), *L. angustifolia* (4.9), *D. moldavica* (4.9), and *A. pseudoalhagi* (42.3). The mixture (200 g) was decocted in boiling water at a ratio of 1:10 (w/v) for 3 h. After filtration, the residue was re-extracted twice for 3 h in the same volume of boiling water. The resulting crude extract was filtered, concentrated using a rotary evaporator under reduced pressure, frozen at –70°C in an ultra-low-temperature freezer, and then pulverized in the freeze dryer. The obtained powder was used for this study. The yield was 49.69% (w/w) relative to the total mass of dry materials. The powder was stored at 4°C until use. The experiment provided three batches of samples for the determination of different polyphenol content.

The powder sample of ASMQ was accurately weighed (5 g), transferred to a 100 mL erlenmeyer flask, and extracted using

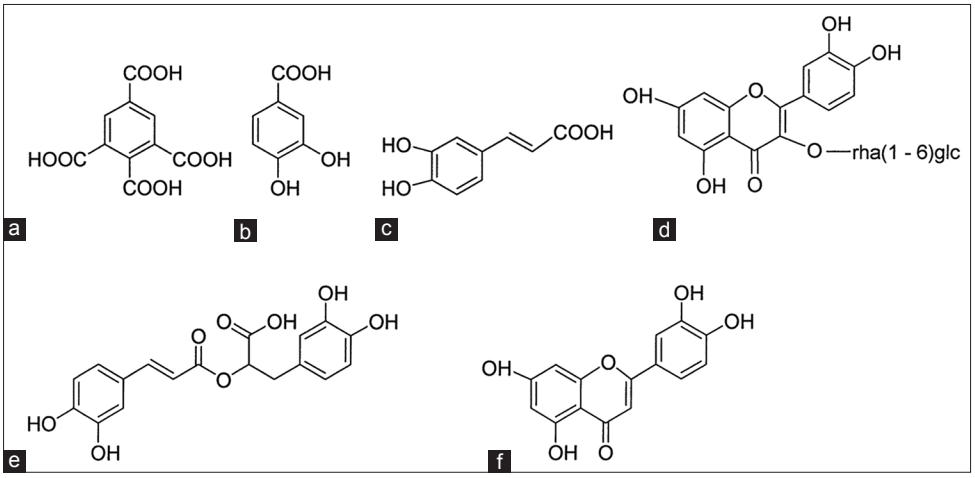

Figure 1: The chemical structures of the nine components: (a) Gallic acid; (b) protocatechuic acid; (c) caffeic acid; (d) rutin; (e) rosmarinic acid; (f) luteolin

50 mL of 75% ethanol solution. The mixed solution was soaked for 30 min, ultrasonicated for 30 min, and filtered through a 0.45 μm membrane filter as the test solution.

Standard gallic acid, protocatechuic acid, caffeic acid, rutin, rosmarinic acid, and luteolin were accurately weighed, dissolved in methanol solution, diluted to appropriate concentrations, and filtered through a 0.45 μm membrane filter. All solutions were stored at 4°C in the refrigerator until analysis.

High-performance liquid chromatography-diode array detector analyses

For quantification purposes, an Agilent 1200 series HPLC instrument (Agilent Technologies, USA) composed of an online degasser (G1322A), a quaternary pump (G1312A), an autosampler (G1367C), a column temperature controller (G1316A), and a DAD (G1315B) was used. Analyses were carried out on an Agilent TC-C_{18} reversed-phase analytical column (4.6 × 250 mm, 5 μm) at a flow rate of 1 mL/min. The detection wavelength was set at 290 nm. The injection volume was 10 μL, and the column temperature was maintained at 30°C. The mobile phase consisted of the solvent A (0.3%, v/v solution of formic acid in water) and solvent B (0.3%, v/v solution of formic acid in methanol) filtered through a 0.45 μm membrane filter by gradient elution as follows: 100% A at 0 min, 92% A at 15 min, 83% A at 30 min, 75% A at 50 min, 55% A at 80 min, 40% A at 100 min. All data were collected and analyzed using Agilent 1200 series HPLC Chemstation Software and Origin (Version 8.0). Figure 2 shows the typical HPLC chromatogram and ultraviolet (UV) spectra of ASMQ. Figure 3a shows the standard chromatogram, and Figure 3b shows the sample chromatogram.

RESULTS

Method development and optimization

To obtain chromatograms with well-resolved peaks and comfortable analysis time/run, chromatographic conditions such as column temperature, type of reversed-phase column, extraction solvent, detection wavelength, and mobile phase were optimized [Figure 2].

Using isocratic elution, the six compounds cannot be effectively separated; thus, gradient elution was used throughout the study. To determine the appropriate wavelength for the simultaneous determination of ASMQ, the HPLC system using six compounds including luteolin, gallic acid, caffeic acid, rutin, and rosmarinic acid as standards. For DAD analysis, the wavelength range was 190-400 nm. All chromatograms and the UV characteristic spectra of the six reference compounds were compared. The six active compounds had higher absorbance, better separation, and steady baseline at 290 nm. Other chromatographic variables were also optimized, including column temperatures (25°C, 30°C, or 35°C) and reversed-phase column types, that is, Agilent TC-C_{18} (4.6 × 250 mm, 5 μm), Aglient XDB-C_{18} (4.6 × 250 mm, 5 μm), and Wondasil C_{18} (4.6 × 250 mm, 5 μm) reversed-phase analytical column. Optimum separation was eventually achieved at 30°C column temperature, and the reversed-phase column type was Agilent TC-C_{18}. ASMQ chromatograms were also obtained at different detection wavelengths.

In this study, using mixtures of methanol and water at different ratios as the mobile phase, satisfactory separation was not achieved. Thus, a small amount of acid was added

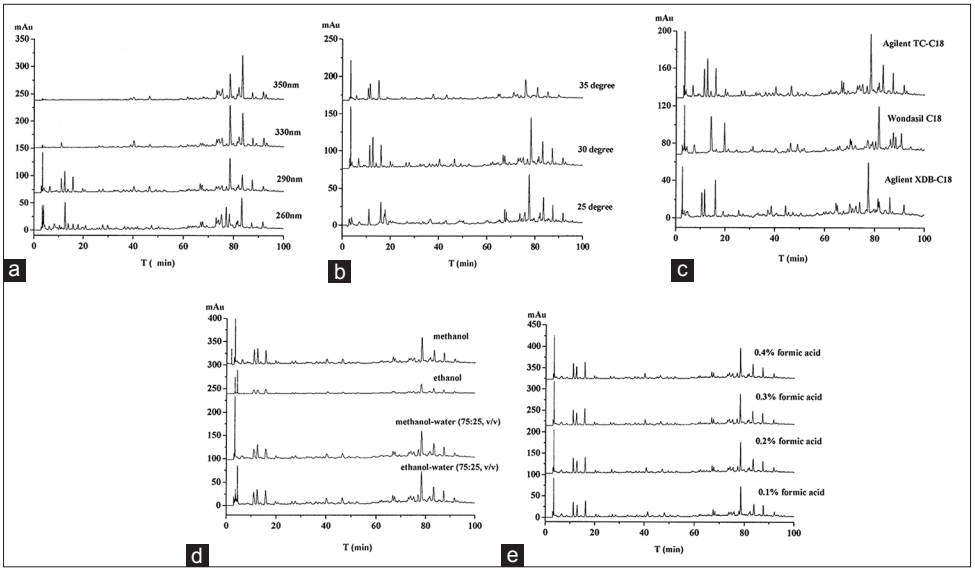

Figure 2: The chromatogram of the extracts of abnormal savda munziq under different detection wavelength (a), column temperature (b), types of reversed-phase column (c), extraction solvents (d), and mobile phase (e)

to the mobile phase. The acid inhibited the ionization of acidic compounds in ASMQ extraction to improve peak shape and restrain peak tailing. We found that the presence of acids during the mobile phase enhanced the resolution. Subsequently, 0.1%, 0.2%, 0.3%, and 0.4% formic acid aqueous solutions were used and compared. Results showed that 0.3% formic acid in the mobile phase significantly improved the retention behavior and peak shape of the different ASMQ components.

Prior to sample analysis, the optimum extraction process had to be investigated. The ASMQ powder sample (5 g) was extracted with 50 mL of solvent in an erlenmeyer flask by sonication for 30 min. The mixture was then passed through a 0.22 μm membrane filter. The extraction efficiency of different solvents including methanol-water (75:25, v/v), methanol, ethanol, and ethanol-water (75:25, v/v) were compared. A mixture of ethanol and water (3:1, v/v) was deemed optimum and thus used for comparative analysis.

The following conditions were found to be optimum for analysis: Mobile phase of solvent A (0.3%, v/v, solution of formic acid in water) and solvent B (0.3%, v/v, solution of formic acid in methanol). Gradient elution was performed as follows: 100% A at 0 min, 92% A at 15 min, 83% A at 30 min, 75% A at 50 min, 55% A at 80 min, 40% A at 100 min, 0% A at 102 min, and 0% A at 112 min. The flow rate was 1.0 mL/min, and the column temperature was 30°C. A typical HPLC chromatogram is shown in Figure 2. The retention times (RT) for the six compounds were 12.458 min (gallic acid), 20.666 min (protocatechuic acid), 40.012 min (caffeic acid), 75.028 min (rutin), 78.799 min (rosmarinic acid), and 97.678 min (luteolin).

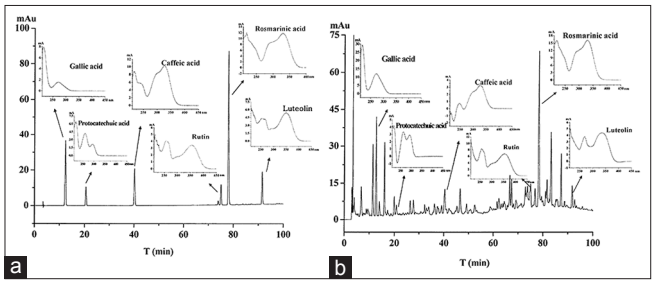

Figure 3: A typical high performance liquid chromatography chromatogram and ultraviolet spectra of abnormal savda munziq. (a) Standard chromatogram; (b) sample chromatogram

System suitability

To ensure adequate performance of the chromatographic system, we evaluated the resolution (R), RT, number of theoretical plates (N), and tailing factor (T) using six replicate 5 mL standard-solution injections. As shown in Table 1, all parameters were within acceptable limits. RT and peak area were checked for repeatability by injecting the standards mixture at a concentration of 0.01 mg/mL into the HPLC system over five runs. The RSDs of both relative RT and relative peak area were < 4.72%.

Validation of the quantitative analysis

Linearity, limit of detection and limit of quantification

After establishing the optimum conditions, method validation was performed. Good linear correlation and high-sensitivity under these chromatographic conditions were confirmed by the correlation coefficients (R^2), limits of detection (LODs), and limits of quantification (LOQs) [Table 2].

Table 1: System-suitability data

	Gallic acid %	Protocatechuic acid %	Caffeic acid %	Rutin %	Rosmarinic acid %	Luteolin %
Resolution (R)	23.54±1.17	15.512±2.38	30.42±4.059	1.854±4.39	5.09±3.44	7.65±3.57
Retention time	12.84±4.72	20.5344±0.35	40.71±1.98	75.55±0.61	78.57±0.45	91.78±0.29
Theoretical plates (N)	10,262±1.96	21,881.4±3.48	51,511±3.88	245,650±1.49	310,791±2.95	349,116.4±7.37
Tailing factor (T)	0.91±1.17	0.902±1.45	0.988±1.81	0.964±2.99	0.98±1.51	0.986±1.670

Mean±RSD. RSD: Relative standard deviation

Table 2: Statistical results of linear regression equation analysis in the determination of the six investigated compounds

Compounds	Calibration curve	Regression coefficient (R^2)	Linear range (μg)	LOD (μg/mL)	LOQ (μg/mL)	Contents (mg/g) ±RSD (%)
Gallic acid	Y=584.4559 X+12.3038	0.9994	0.3940-2.3640	0.015	0.045	0.4810±024
Protocatechuic acid	Y=480.3150 X+5.882	0.9996	0.1226-0.7356	0.004	0.012	0.0675±0.20
Caffeic acid	Y=1247.5926 X–0.2641	0.9991	0.1216-0.7296	0.056	0.168	0.1196±037
Rutin	Y=215.1735 X−0.2403	0.9990	0.3080-1.8480	0.039	0.131	0.4370±13
Rosmarinic acid	Y=703.5375 X+4.0915	0.9995	0.7000-4.2000	0.043	0.155	0.8360±021
Luteolin	Y=424.6868 X−2.4477	0.9991	0.2300-1.3800	0.022	0.074	0.1980±12

LOD: Limits of detection; LOQ: Limits of quantification; RSD: Relative standard deviation

The correlation coefficient R^2 and linear regression equations were analyzed. The linearity calibration curves were plotted based on at least five chemical marker calibration points performed in triplicate. A linear regression equation was $Y = AX + B$, where Y is the peak area, X is the concentration of the standard compound (μg), A is the slope rate of the line, and B is the intercept of the straight line with the y-axis. Results of regression analyses and the calculated correlation coefficients (R^2) are listed in Table 2. The high-correlation coefficient values ($R^2 > 0.999$) indicated good linearity between peak areas (Y) and compound concentrations (X, mg/g) within relatively wide concentration ranges. High-regression coefficients for the calibration curves were obtained for all investigated compounds at the wavelengths where the compounds had responses, except for gallic acid, protocatechuic, acid caffeic acid, rutin, rosmarinic acid, and luteolin that were tested only at 290 nm.

Limits of detection and LOQ under the present chromatographic conditions were defined as the lowest concentration with the signal-to-noise ratio of 3 and 10 as criteria, respectively. The LODs and LOQs for the six chemical components are also listed in Table 2.

Precision, repeatability, stability and recoveries

Precision was evaluated by analyzing standard samples. Thus, six individual sample solutions were analyzed. The RSD of the mean content for each compound was calculated and ranged from 0.13% to 0.60% for intraday precisions. The results are listed in Table 3.

Extraction reproducibility was also investigated for the six components by comparing six samples from six independent extractions. RSDs were calculated to measure the method reproducibility. Results indicated that the RSDs of all detected compounds were <0.72%, which indicated that the develop method had good reproducibility.

For stability tests, ASMQ samples were analyzed at 0, 2, 4, 8, 12, and 14 h at room temperature. Stable RSDs of the sample solution were found to have mean content values <0.21%, as shown in Table 3.

Recoveries of the six compounds were determined by adding known contents of standard samples (50%) to the known amounts of ASMQ samples and then comparing the determined amount of these standards with the amount originally added [Table 3]. The mixture was extracted and quantified as described above. The mean recovery of the method ranged within 99.39-104.85% with RSDs < 0.85%.

Results indicated that the HPLC method had good precision, repeatability, stability, and recovery [Table 3] and that the developed assay was reliable and useful for assessing ASMQ quality. The developed analytical method was also reproducible, highly precise, and thus satisfactory for quantitative analysis.

Sample analysis

The proposed HPLC analytical method was successfully used to simultaneously determine six components in ASMQ samples. Quantitative analytical results indicated that the six components of their contents were substantial. The peaks in the chromatograms of each sample were identified by comparing RT s and UV spectra with authentic standards. The content of the investigated plant phenolic compounds in crude extracts was expressed as the mean mg/g extract ± standard deviation [Table 2]. In the crude ethanol-water (75:25, v/v) extract of ASMQ, the following compounds were identified and quantified: Gallic acid, protocatechuic acid, caffeic acid, rutin, rosmarinic acid, and luteolin. Among the quantified compounds, gallic acid, rutin, and rosmarinic acid were the most abundant.

DISCUSSION

In conclusion, an Agilent TC-C18 reversed-phase HPLC-DAD method was successfully used to simultaneously identify six phenolic compounds in ASMQ. The results elucidated the phenolic constituents of ASMQ. Quantitative data showed that gallic acid, rutin, and rosmarinic acid were the main phenolic constituents of ASMQ. We provided a substantial basis for further research on the quality control and clinical application of ASMQ. The phenols identified in ASMQ can be

Table 3: Precision, repeatbility, stability and recovery results for the assay of the 6 analytes

Analyte	Precision		Repeatability		Stability		Recovery[a]	
	Mean (mg/g)	RSD[b] (%)	Mean (mg/g)	RSD[b] (%)	Mean (mg/g)	RSD[b] (%)	Recovery (%)	RSD[b] (%)
Gallic acid	587.661	0.26	574.604	0.13	583.154	0.033	99.81	0.25
Protocatechuic acid	158.444	0.60	63.037	0.72	70.319	0.21	104.85	0.15
Caffeic acid	380.893	0.13	292.910	0.18	296.647	0.029	103.2	0.26
Rutin	168.022	0.34	179.107	0.25	187.733	0.083	101.71	0.85
Rosmarinic acid	1237.088	0.07	1164.994	0.032	1179.725	0.18	100.06	0.067
Luteolin	285.762	0.37	165.800	0.49	165.671	0.11	99.39	0.66

[a]Recovery (%)=(Detected amount−original amount)/spiked amount×100; [b]RSD (%)=(SD/mean)×100. RSD: Relative standard deviation; SD: Standard deviation

considered chemical markers of this species, which may be the major bioactive constituents of ASMQ. Given availability, rapidity, and reliability of the developed method, it can be used to analyze and effectively control the quality of AMST.

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