IDENTIFICATION OF KURARINONE BY LC/MS AND INVESTIGATION OF ITS THERMAL STABILITY

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ABSTRACT

A herbal ingredient kurarinone (7, 2', 4'-trihydroxy-8-lavandulyl-5-methoxy flavanone) was isolated from the roots of *sophora flavescens* Ait. by ultrasonic extraction, which is one of traditional Chinese medicine (TCM) materials. A reliable high performance liquid chromatography with mass spectrometry (HPLC-MS) method was used for determination of kurarinone. C18 column (150×4.6 mm, 5 μm) and methanol-water mobile phase (70:30, by volume) were used in the chromatographic separation of active components from the herb. HPLC-ESI-MS (UV, 288 nm; m/z, 437.5 [M-H]⁻) was adopted for kurarinone identification. Kurarinone with purity of 95.5% was obtained by silica gel column followed by reverse-phase column chromatography. The thermal stability of kurarinone was investigated, which illustrates that kurarinone was stable below 25 °C in 48 h, but a degradation of 25.54% was observed at 80 °C for 2 h.

Keywords: *Sophora flavescens* Ait; Leguminosae; Flavonoids; Kurarinone; HPLC-ESI-MS; Ultrasonic extraction; Thermal stability.

INTRODUCTION

There has been growing interest over the past 20 years in the natural medications because of their low toxicity and effective therapeutical performance. Increasing evidence illustrates that flavanones are beneficial to human health. The roots of *sophora flavescens* Ait. are used as traditional Chinese medicine (TCM) material effective in antipyretic, analgesic, anthelmintic and stomachic¹. Kurarinone (7, 2', 4'-trihydroxy-8-lavandulyl-5-methoxy flavanone, Fig. 1), as a natural pigment derivative from the herb *S. flavescens*², has been proved to be effective on anti cancer, anti androgenic³, anti atherosclerotic⁴, and inhibition of tyrosinase⁵ in recent years.

Fig. 1. Full scan mass spectrum of kurarinone and its chemical structure (molecular weight 438).

Techniques of maceration and hot extraction in Soxhlet apparatus are widely used to obtain plant extracts. However, the herbal ingredient may be degraded for long-duration heat treatment⁶. The efficiency of extraction is influenced by solvent type, solvent quantity, extraction temperature, duration and cycles⁷. Ultrasonic can improve the rate, selectivity and yield in extracting active components⁸. A reverse-phase liquid chromatography (LC) with methanol-water (70:30, v/v) as mobile phase was used to isolate kurarinone and UV at wavelength 290 nm was selected for its detection⁴. Analyses of flavonoids with ESI-MS⁹ in *S. flavescens* have been reported by a paper⁹ which gives the formulae of fracture mode of flavonoids in *S. flavescens*, illustrated in Fig. 2. A total of 24 flavonoids including kurarinone are identified¹⁰, but their fragment ions are not consistent with the formulae reported in Bai's work⁹.

Fig. 2. The formulae of bond cleavages of flavonoid from *S. flavescens* based on Bai's work.

The major aim of this study was to verify the formulae of fracture mode of flavonoids in *S. flavescens*. In addition, the process to obtain high purity kurarinone from *S. flavescens* roots, thermal stability of kurarinone at different temperatures and the advantage of the ultrasonic extraction compared with maceration were investigated.

EXPERIMENTAL

Materials

S. flavescens radix samples (dry) purchased from the herbal store in Shuanghe Pharmaceutical Co. (Taiyuan, China) was identified by Professor J.P. Luo in Shanxi Institute of Pharmaceutical and Biological Products Controlling. Ultrasonic equipment (As2060 B) was from Autoscience Instrument Co. Ltd (Shanghai China). The authentic kurarinone was extracted from *S. flavescens* by us and its structure was fully characterized; the purity was above 95.5% as determined by HPLC with the UV detector at three different wavelengths (270, 288 and 320 nm). Vacuum desiccator was ZK-82A from Shanghai Laboratory

Instrument General Works Co., Ltd (Shanghai China). Ethyl acetate, petroleum ether, acetone, ethanol, n-butanol and n-hexane were of analytical grade from Tianda Chemical Reagent Co., Ltd (Tianjin China). Methanol is HPLC grade is from Tianjin Kermel Chemical Reagent Co., Ltd (Tianjin China). Re-distilled water was made in our laboratory.

Sample preparation

Samples for recovery

In order to investigate the reliability of HPLC for kurarinone detection, these assay trials were carried out. The sliced roots of *S. flavescens* were ground into powder and then vacuum-dried for further processing. Dried powder (0.6 g) added x ml ($x = 0, 1, 2, 3$) standard solution (2.28 mg/ml, details in 2.4 samples for liquid chromatography - mass spectrometry) was extracted with 100 ml ethanol for 1 h at room temperature (25 °C) with ultrasonic equipment. The residue was washed with 20 ml ethanol for 3 times. Extracts and washing solution were combined and filtered with a membrane filter of 0.45 μ m and added ethanol to 200 ml. Solution (5 ml) was diluted to 25 ml for analysis. Every group of this trial was repeated 3 times and the final added concentration of kurarinone were 0, 2.28 μ g/ml, 4.56 μ g/ml, 6.84 μ g/ml.

Samples for thermal stability

In order to study the thermal stability 0.6 g dried powder was extracted with 100 ml ethanol for 1 h at room temperature with ultrasonic equipment. The residue extracted by ethanol was washed by 20 ml ethanol for 3 times. Extracts and washing solution were combined and filtered and added ethanol to 200 ml. Solution (5 ml) was diluted to 25 ml for analysis. This experiment was repeated 15 times.

Samples for comparisons of extractive efficiency

For comparisons of extractive efficiency between ultrasonic extraction and maceration with different parameters, four groups of samples were prepared. Dried powder (0.6 g) was ultrasonic extracted with 100 ml ethanol for 1 h at room temperature (25 °C). The residue was washed by 20 ml ethanol for 3 times. Extracts and washing solution were combined and filtered and added ethanol to 200 ml. Solution (5 ml) was diluted to 25 ml for analysis. The next 3 groups of trials were carried out in the following extraction conditions: ultrasonic extraction (2 h) and maceration (1 h, 6 h). All of the following trials were repeated 2 times.

Samples for Liquid chromatography - mass spectrometry

To prepare authentic standard kurarinone sample, 2 kg dried powder of *S. flavescens* was first extracted with 2 l petroleum ether at room temperature to decrease for 3 times, each time for 2 h. The degreased powder was vacuum-dried and extracted with 5 l ethanol for 3 times (each time for 40 min). The extracts were combined and filtered. The filtered extract was concentrated to 1 l, added 2 l re-distilled water successively and placed still for 1 h. After that, the extracts were filtered and the residue was washed with distilled water (3×0.3 l). The solid filter cake was triturated into powder. The powder was partitioned between water (3×1 l) and n-hexane (3×1 l), and the water phase was extracted with ethyl acetate (3×1 l) and n-butanol (3×1 l) in turn. The ethyl acetate extract (20 g) was subjected to silica gel column chromatography (60–100 mesh, 0.9 kg) with a gradient of acetone/ethanol (50:1, 30:1, 20:1, 10:1, 7:1, v/v, each 1 l) as mobile phase, and 5 fractions were washed down. Fraction 4 (10:1, 2 g) was rechromatographed over reversed phase column chromatography (50 g, YMC-Gel C18, 50 μ m), eluted with methanol/water (50:50, 60:40, 70:30, 85:15, 90:10, 100:0, each 200 ml). Yellow amorphous powder (fraction F-4-3, 70:30, 0.3 g) was prepared and then recrystallized in methanol/water (80:20, 20 ml, 5 °C); a pale yellow powder (0.1 g) was then obtained. 5 mg standard sample (named β) was then dissolved in 50 ml ethanol for HPLC-MS analysis.

Standard sample 22.6 mg was dissolved in 100 ml ethanol, and the solution was diluted to 2.28, 4.56, 9.12, 18.24, 22.8 μ g/ml to establish a standard curve, $R^2 = 0.9985$. Every concentration of sample was in this range.

HPLC analysis

A high performance liquid chromatograph (HPLC) coupled with a UV-vis multi-wave-length detector (LC-10ATVP Shimadzu) was used. The separation was achieved on a Shimadzu 5 μ m ODS 4.6 $\times$ 150 mm column at 32 °C. The mobile phase was methanol-water (70:30) at a flow rate of 1.0 ml/min with an injection volume of 10 μ l. The monitoring wavelength was 288 nm.

Liquid chromatography - mass spectrometry

An Alliance 2690 separation module with auto sampler and quaternion

gradient pump (Waters, USA.) was used for HPLC separation and Micromass ZMD4000 with ESI (negative mode) interface were used for mass spectrometry.

HPLC: The chromatographic column was a 2.1 $\times$ 150 mm Symmetry C18 with 3.5 μ m size (Waters, USA.). The mobile phase was methanol (HPLC grade, Dikma)–re-deionized water (70:30) at a flow rate of 0.2 ml/min, filtered with a Millipore 0.45 μ m filter before used. The injection volume was 10 μ l.

ESI-MS: High purity nitrogen was used as the auxiliary gas. The operating parameters of MS were desolvitized gas, 360 l/h; cone gas, 104 l/h; Cone voltage, 25V; capillary voltage, 3.5 kV; extractor voltage, 5 V; source block temperature, 100 °C; and desolvation temperature, 250 °C. Mass spectral data were collected in the scan range of m/z from 100 amu to 1000 amu. All LC-MS data were processed by MassLynx™ data acquisition software.

Recovery

Four groups of samples were injected to HPLC for detection. The first group of samples which did not add standard solution was blank control group. Detections of the other three groups of samples were compared with the blank, and then the recovery rate was obtained, listed in Table 1.

Thermal stability

Samples were placed in water bath for 2 h at 50 °C, 60 °C, 70 °C and 80 °C, respectively. After cooling down to 25 °C, they were diluted with ethanol to 25 ml and then analyzed with HPLC. To observe the stability of kurarinone at room temperature, one group of samples were also analyzed after keeping at 25 °C for 48 h. Every group was repeated 2 times.

RESULTS AND DISCUSSION

Standard sample β was identified with HPLC-ESI-MS, which started with a full scan (from m/z 100 to m/z 1000) of 1 μ g/ml solutions of the powder in negative ion modes. A typical mass spectrum of standard kurarinone is shown in Fig. 1 (ESI: m/z 437.6 $[M-H]^-$) and the full scan of UV spectra is shown in Fig. 3. There are seven significant fragments in the spectra: 876.0, 875.0, 438.5, 437.6, 275.3, 161.6 and 137.3 labeled in Fig. 1. The m/z value at 876.0 and 875.0 were two dimeric-compounds formed in ESI experiment and m/z value 438.5 was isotopic ion. The m/z 437.6 was quasi-molecular ion. The fractures mode of other three spectra: 275.3, 161.6, 137.3 are shown in Fig. 1 and the formulae of bond cleavages are consistent with Bai's work⁹. In published reference¹⁰, ESI-MS² was used, the m/z values were 421 ($[M+H-H_2O]^+$), 315 ($[M+H-lanandulyl]^+$), 303 ($^{13}A^+-H_2O$), 297 ($[M+H-lanandulyl-H_2O]^+$), 179 ($^{13}A^+-lanandulyl$) and group lanandulyl in his work might be lavandulyl^{14,9,10}. These results of m/z values did not consistent with reference⁹ and the current work. The optimal UV detection wavelength was 288.5 nm according to Fig. 3, and UV of 288 nm was then adopted for HPLC detector in this work.

Fig. 3. Full scan of UV spectrum of kurarinone.

The chromatograms of standard sample β are shown with the UV detection wavelength 288 nm, 270 nm and 320 nm, respectively. In all spectra, the principal peak accounted for 95.5% of total peak area, as shown in Fig. 4. There are no obvious interfering peaks, which indicate that the purity of sample β is almost 95.5%. This proves that the process to obtain high purity kurarinone is feasible.

Fig. 4. HPLC chromatogram of kurarinone standard sample (β) (4.56 $\mu\text{g/ml}$) with UV detector at three different wavelengths of (a) 270 nm, (b) 288 nm, (c) 320 nm.

There are no interfering peaks present near the principal peak of kurarinone, which implies the separation of kurarinone and other components was good. In this work, kurarinone was examined in linear ranges of 2.28–22.8 $\mu\text{g/ml}$. The extractive recoveries of kurarinone were 98.7–102.9%, RSD 2.09%, as listed in Table 1. All of these show that the current HPLC method is effective in analysis of kurarinone.

Table 1. Extraction recovery of kurarinone.

Concentration of added kurarinone ($\mu\text{g/ml}$)	Observed concentration ($\mu\text{g/ml}$)	Recovery (%)	RSD (%)
0	5.91	–	–
2.28	8.16	98.7	2.09
4.56	10.67	104.4	
6.84	12.93	102.6	

Every group was repeated 3 times, and mean value was adopted (RSD < 4.31%). Recovery = [(observed mass concentration – basal mass concentration) / (mass concentration of added kurarinone)] $\times$ 100%.

A comparison of extractive efficiencies among different treatments (ultrasonic extraction and maceration) is listed in Table 2. The relative extraction ratio of ultrasonic method is 81.4% versus that of maceration with 58.6%, in the same period of 1 h. The relative extraction of ultrasonic for 2 h reached 97.4%; in contrast to that, it is only 85.7% by maceration for 6 h. The relative recovery with maceration increased from 58.6% to 85.7% with the extraction time from 1 h to 6 h. In contrast, ultrasonic extraction is not evident increase to prolong extraction time. High frequency ultrasonic wave propagation can destruct the pores so the solute can infiltrate in a short period of time⁷. All of these illustrate that ultrasonic extraction is much more effective than maceration in extracting of kurarinone in *S. flavescens*. The frontier one can improve extraction rates and avoid using heat.

Table 2. Extraction rates of kurarinone with different methods.

Extracted method	Recovery ratio (mg/g)				RSD (%)	Relative recovery ratio (%)
	1	2	3	Mean		
Ultrasonic (1 h)	5.68	6.03	5.93	5.88	2.27	81.4
Ultrasonic (2 h)	7.11	7.05	6.91	7.03	1.07	97.4
Maceration (1 h)	3.92	4.24	4.52	4.23	4.84	58.6
Maceration (6 h)	6.14	6.13	6.29	6.19	1.11	85.7

The content of kurarinone in the dried powder was 7.22 mg/g. Relative recovery ratio = (recovery ratio/7.22) $\times$ 100%.

The thermal stability of kurarinone at different temperatures was examined, as shown in Table 3. All of the samples were in volumetric flask and placed in ultrasonic equipment to eliminate the air for 30 min. Therefore, oxidation reaction of kurarinone was eliminated. It was reported that phumbagin had certain degradation when exposed at high temperature for a certain time⁶. Our results illustrate that kurarinone is stable at 25 °C at least for 48 h. A very low decrease in the mass concentration of kurarinone was observed when the temperature is below 50 °C (holding for 2 h). However, with increasing temperature up to 60 °C and 70 °C, the decreases in the mass concentration of kurarinone are evident reached 15.96% and 19.51%. At 80 °C, the decrease ratio is even up to 25.54%. The test of thermal stability illustrates that kurarinone should be conserved at a temperature below 25 °C.

Table 3. Decrease ratio of kurarinone at different temperatures

Temperature (°C)	25	50	60	70	80
Duration (h)	48	2	2	2	2
Mass concentration ($\mu\text{g/ml}$)	20.5	20.0	17.2	16.6	15.3
Decrease ratio (%)	0	2.6	16.0	19.2	25.5

Data expressed as mean values with the RSD < 3.28% (n=3).

CONCLUSIONS

A herbal ingredient kurarinone (7, 2', 4'-trihydroxy-8-lavandulyl-5-methoxy flavanone) was isolated from the root of *S. flavescens*. A reliable HPLC-MS method developed to determine kurarinone and the results are consistent with Bai's work⁹ but different with Zhang's¹⁰. Ultrasonic extraction is much more effective than maceration in extracting kurarinone from *S. flavescens*. The kurarinone sample with content of 95.5% was obtained by silica gel column followed by reverse-phase column. The thermal stability tests illustrate that kurarinone is stable below 25 °C, but a degradation of 25.54% is observed at 80 °C.

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