Printed in the Republic of Korea
ANALYTICAL SCIENCE & TECHNOLOGY
Vol. 30 No. 2, 82-88, 2017
https://doi.org/10.5806/AST.2017.30.2.82

Development of simple HPLC-UV method for discrimination of Adenophorae Radix

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(Received February 14, 2017; Revised April 11, 2017; Accepted April 11, 2017)

Abstract: Adenophorae Radix (AR) is a frequently used medicinal herb; because of its popularity, products containing similar herbal products are often sold as substitutes, especially if their morphology is similar. However, any analytical method to identify AR based on quantitative analysis is not registered in Korea, Japan and China Pharmacopoeias. This study developed a simple HPLC method to discriminate between authentic AR and substitutes. Linoleic acid was used as a marker compound of AR. Our optimized HPLC-UV conditions included a mobile phase of 90 % acetonitrile under isocratic condition, and a flow rate of 1.0 mL/min at room temperature. Detection wavelength was set at 205 nm. Linoleic acid was detected at 13.5 minutes for a total analysis time of 20 minutes. The standard herb of AR contained 0.025 % of linoleic acid, while four authentic AR samples and eight substitutes contained 0.040~0.071 % and 0.004~0.014 %, respectively. Comparison of the linoleic acid concentrations of the sample types to reference AR showed that among 12 samples, only the four samples were authentic. Thus, our HPLC-UV method, along with our suggested content criterion for linoleic acid concentration, can be used for the quick and accurate determination whether the herbal products are authentic AR or substitute.

Key words: Adenophorae Radix, HPLC-UV, linoleic acid, discriminant analysis

1. Introduction

In recent decades, herbal medications have become more important with respect to drug development and discovery, as many have valuable inherent bioactivities. Interestingly, many species with similar morphologies have different bioactivities. The mislabeling of herbs or herbal medicines can give rise to unwanted outcomes, including ineffective treatment or serious adverse effects. The composition of herbal materials can vary by species, making it necessary to control the quality of these materials to detect adulterants, as well as to avoid potentially harmful health effects.[1]

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Adenophorae Radix (AR) is defined as the dried root of two species - *Adenophora triphylla* var. *japonica* Hara and *Adenophora stricta* Miq in The Korean Herbal Pharmacopeia.[2] In Chinese Pharmacopoiea, the species of AR is defined as *Adenophora tetraphylla* and *Adenophora stricta* Miq.[3] These species belong to *Adenophora* genus of the Campanulaceae family.[2] *Adenophora triphylla* is distributed widely in Korea, Japan, China, Taiwan and Russia,[3] while *Adenophora stricta* is found in China, Korea and Japan.[4] In medicinal form it is known as 'Sha shen' in China and commonly used to treat whooping cough and chronic bronchitis.[5] In The Pharmacology of Chinese herbs, AR contains saponins and it has high hemolytic activity, antibacterial properties and stimulates myocardial contractions.[5] In Korea, AR is used widely to treat asthma.[6] Recently reported bioactive properties of AR include anti-inflammatory, anti-asthmatic,[6] anti-oxidation,[7] anti-cancer and anti-melanogenesis.[8,9]

AR is one of the most widely consumed herbs in Korea.[10] Therefore, discriminating between products that contain genuine AR versus those that contain various adulterants is important, especially given that *Adenophora* genus encompasses 62 species in East Asia,[4] all of which have similar root shapes. The morphology of Glehniae radix (Apiaceae family) is close to that of AR,[11] while roots from *Codonopsis lanceolata* Trauty are used as an AR substitutes known commercially as 'white AR'.[2] Many species and varieties of *Adenophora* and *Glehnia* (both of which are not easily discriminable from AR based on appearance or morphology) are available on the East Asia commercial market as AR substitutes or adulterants.[12]

Recently, DNA sequencing analysis was applied to discriminate between AR and its various adulterants[13] however, this technique is too complex to routinely identify AR on a commercial basis. High performance liquid chromatography (HPLC) analysis of AR has been accomplished using (6*R*,7*R*)-*E*,*E*-tetradeca-4,12-diene-8,10-diyne-1,6,7-triol as a marker compound.[14] This compound, however, is present at low concentration in AR and cannot be purchased on the standard market, making it unsuitable as a standard method of commercial AR analysis. Another study used gas chromatography coupled with flame ionization detection to quantify *β*-sitosterol and lupenone in *A. triphylla*;[15] this method necessitated complicated sample preparation to increase the volatility of the constituents, and thus also the resolution. Consequently, no convenient HPLC method has been developed to discriminate between AR and its adulterants, to allow control of the quality of commercial AR products available on the market. A fast and simple HPLC method could be one means of discriminating authentic AR products, which could be applied easily by pharmaceutical companies.

AR contains triterpenoids (including lupenone, lupeol, taraxerol,[16,17] methyl adenophorate, and triphyllol),[18] steroids (including *β*-sitosterol, and *β*-sitosteryl glucoside),[18] phenolic glycosides (including she-shenoside, andsiringinoside)[19] and linoleic acid.[19] No commercial standards exist for methyl adenophorate, triphyllol, sheshenoside and siringinoside. The other penta-cyclic skeleton compounds (including lupenone, lupeol and taraxerol) do not have chromophore group therefore, UV detection intensity of these compounds is poor. Thus, linoleic acid is potentially a good marker compound for fast and convenient commercial analysis of AR. This is because the analysis of linoleic acid does not require complicated sample preparation to enrich the analyte. Furthermore, this compound is widely available and thus inexpensive. Therefore, we developed and validated a precise and robust high performance liquid chromatography-ultraviolet (HPLC-UV) method that uses linoleic acid content to quantify AR, as well as to detect AR adulterants commonly available in the market.

2. Experimental

2.1. Chemicals and samples

Linoleic acid was obtained from Sigma Chemicals (St. Louis, MO, USA) and HPLC grade acetonitrile, methanol, and ethyl alcohol were from Burdick & Jackson (Muskegon, MI, USA). Reference AR were

purchased from the Korean Ministry of Food and Drug Safety. Four samples were collected in China (S1, S3, S5 and S7), and eight in Korea (S2, S4, S6, S8, S9, S10, S11 and S12), for blind testing to discriminate between authentic and substitue samples. All samples were stored at 4 °C before use.

2.2. Preparation of samples and standard solutions

AR powder was extracted using different methods (including reflux and ultra-sonication extraction (UE)), with different solvents (ethanol and methanol) used to determine the optimal extraction conditions. First, 20 mL of solvent was added to a 100 mL glass flask containing the sample (1.0 g). Reflux extraction (RE) and UE were performed at 50 °C for 60 min; UE was done at 250 W and 40 kHz. The loss of solvent during extraction was compensated for by using the same extraction solvent. Water and various concentrations of ethanol and methanol, were used to select the optimal extraction solvent.

To prepare standards, 1.0 mg of linoleic acid was dissolved in 1.0 mL of methanol. This standard stock solution was diluted appropriately to create a calibration curve encompassing the concentrations used within our method. All standard solutions were stored in brown bottles at 4 °C until analysis.

2.3. HPLC and ESI-MS conditions

HPLC analysis (LC-20AD series; Shimadzu, Japan) was performed with a UV detector that uses a flow rate of 1 mL/min at room temperature and a Hector C_{18} column (250 × 4.6 mm, 5 μm; RStech, Korea).

To identify linoleic acid, liquid chromatography–tandem mass spectrometry (LC-MS/MS) (8040 Series; Shimadzu) apparatus equipped with a photodiode-array detector (PDA) was used. Electrospray ionization (ESI) was operated in negative mode with an interface voltage of -3.5 kV. The de-solvation line temperature was set at 250 °C, with a heat block temperature of 400 °C, and a nebulizing gas flow rate of 3 L/min. Separation was performed using a Phenomenex C_{18} column (150 × 2.0 mm, 5 μm, Phenomenex, USA) at a flow rate of 0.3 mL/min. Detection was first carried out using the PDA detector, and then with the mass spectrometer. The analytical conditions were otherwise the same as for HPLC-UV analysis. Linoleic acid in 12 samples was identified by comparing retention times, as well as UV and mass spectra, to those of the linoleic acid standard.

2.4. Method validation

We validated the linearity, limit of detection (LOD), limit of quantification (LOQ), precision, accuracy and repeatability of our HPLC method. Seven concentrations of linoleic acid (0.0125, 0.025, 0.05, 0.1, 0.2, 0.4 and 0.8 mg/mL) were analyzed in triplicate, and linearity was verified using correlation coefficients. The LOD was estimated at a signal-to-noise ratio of 3:1, and the LOQ was estimated at a ratio of 10:1. Intra-day precision and accuracy were determined by analyzing three concentrations of standard solution five times and precision was expressed RSD. Analysis was carried out over 5 consecutive days for inter-day variability. Repeatability was estimated through analysis of five samples replicates from the same batch; we use three batches (S3, S5 and S7) for this investigation.

2.5. Statistical analysis

The quantification of linoleic acid in the 13 samples was estimated by HPLC-UV using a calibration curve prior to the statistical analysis. Common AR adulterants were identified within samples by comparing their linoleic acid content to that of authentic samples (collected in China). Authentic and inauthentic samples were distinguished by agglomerative hierarchical cluster (HCluster) analysis using Ward's method[19] in Minitab statistical software (ver. 17; Minitab Inc., State College, PA, USA).

3. Results and Discussion

3.1. Chromatographic separation

Linoleic acid is a long chain fatty acid that contains only one carboxylic group, and is thus non-polar. Hence, we used a strong solvent to elute linoleic acid from C_{18} column, and analysis with

90 % acetonitrile in water produced the best separation over a short period. The detector was set at short UV wavelength (205 nm) to detect linoleic acid; this compound is absorbent at these wavelengths due to the presence of two olefinic double bonds within its structure, which yields a low baseline and avoids the UV acetonitrile cut-off at 190 nm.

3.2. Identification and analysis of linoleic acid in AR

Linoleic acid from 12 herbal samples was analyzed using HPLC and LC-MS, and identified by comparing retention times and MS and UV spectra with a linoleic acid standard. Linoleic acid in AR samples and standard solution was eluted at 13.5 min providing the same retention time (*Fig.* 1). Furthermore, the negative-molecular ion at *m/z* 279 $[M-H]^-$ in LC-MS spectrum of peak at 13.5 min indicated that the separated compound from sample is linoleic acid (*Fig.* 2).

3.3. HPLC method validation

We validated our proposed HPLC method according

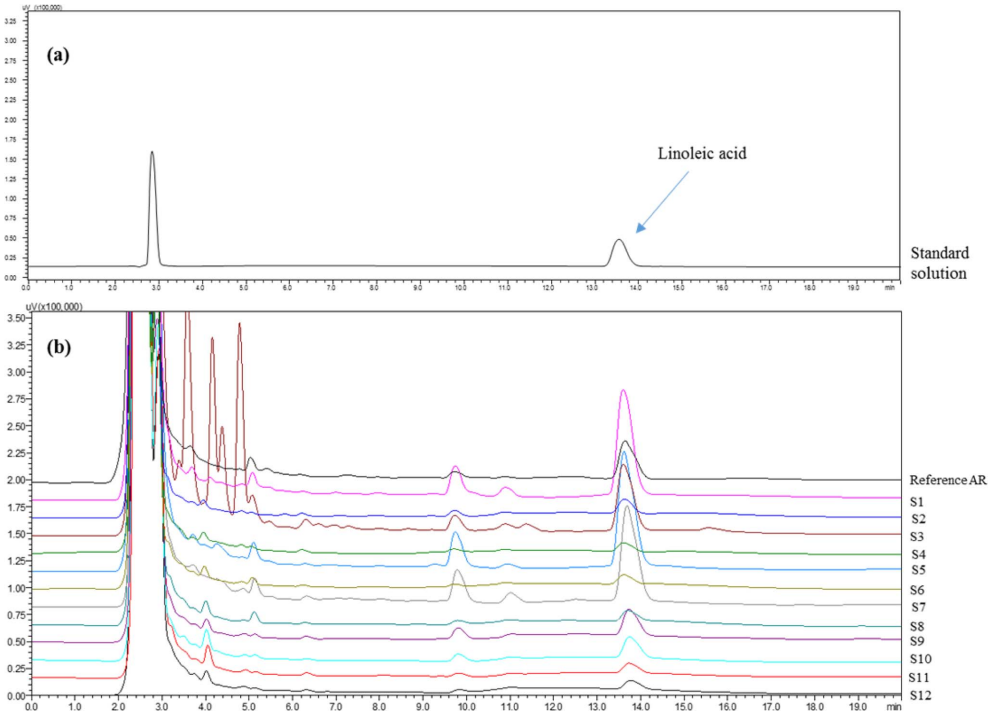

Fig. 1. HPLC chromatogram of (a) standard solution (1.0 mg/mL linoleic acid) and (b) samples (reference AR and 12 samples). HPLC condition: Mobile phase; 90 % acetonitrile, flow rate; 1.0 mL/min, detection; UV 205 nm.

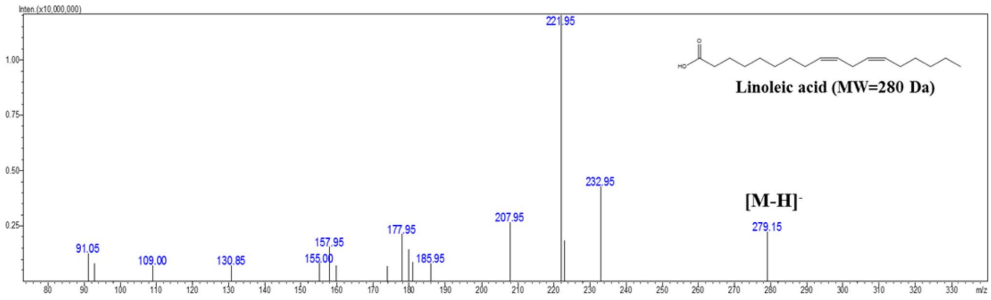

Fig. 2. LC-ESI-MS spectrum of linoleic acid peak in sample at negative mode.

Table 1. Intra-/inter-day accuracy and precisions of analysis for linoleic acid (n=5)

Conc. (μg/mL)	Intra-day			Inter-day		
	Found (μg/mL)	Accuracy (%)	RSD (%)	Found (μg/mL)	Accuracy (%)	RSD (%)
12.5	12.5	99.6	0.4	12.6	101.1	1.5
100.0	102.1	102.1	0.3	102.3	102.3	0.6
800.0	809.7	101.2	0.5	817.4	102.2	1.2

to linearity, LOD, LOQ, intra- and inter-day precision, accuracy, and repeatability. The calibration curve was linear (R^2 = 0.9999) in the range 12.5~800.0 μg/mL, with an LOD of 20 ng/mL and LOQ of 62 ng/mL. *Table* 1 shows the precision (through RSD values) and accuracy at three concentrations. Intra- and inter-day precision of the concentration of linoleic acid ranged from 0.3 %~0.5 % and 0.6 %~1.5 %, respectively, which satisfies the criteria of the Korean Ministry of Food and Drug Safety (less than 5 %).[21] The intra- and inter-day accuracy ranged from 99.6 % to 102.3 %; repeatability was verified by comparing relative peak areas and retention times for linoleic acid over five continuous injections of each authentic batch (S3, S5 and S7). All three batches showed good repeatability based on RSD values for peak areas (≤ 1.4 %), with a retention time variation of less than 0.2 min (*Table* 2). These results confirm that the developed HPLC method performs adequately.

3.4. Optimizing extraction conditions

The difference in extraction efficiency between reflux and ultrasonic procedures was compared using different solvents, including ethanol and methanol

Table 2. Repeatability of injections for three authentic samples (n=5)

Samples	Linoleic acid			
	Peak area		Retention time	
	Area ($\times 10^3$)	RSD (%)	Time (min)	RSD (%)
S3	1600	1.4	13.3	0.5
S5	2466	0.6	13.1	0.2
S7	1941	0.8	13.1	0.1

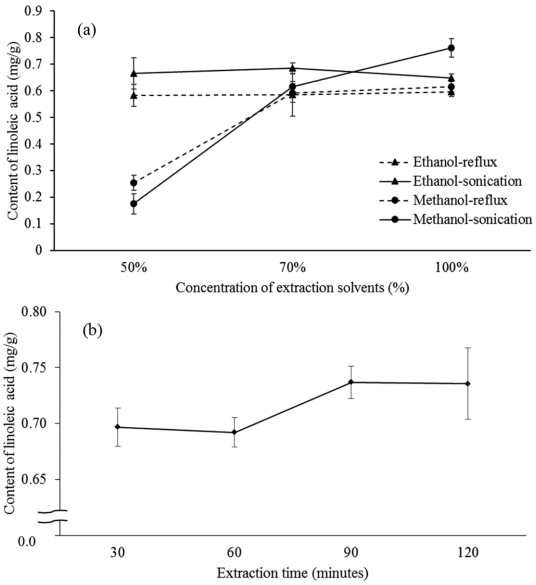

Fig. 3. Effect of (a) extraction solvent and (b) time on the extraction of linoleic acid in Adenophorae Radix samples.

(at 50 %, 70 % and 100 %). Ethanol and methanol have different viscosities (1.074 mPa.s and 0.544 mPa.s, respectively),[22] where methanol had greater extraction efficiency than ethanol. Because of this lower viscosity, methanol can easily enter plant cells during sonication and destroy cell walls. As shown in *Fig.* 3, the concentration of ethanol did not affect the extraction efficiency of linoleic acid (~0.6 mg/g for extraction using both reflux and sonication procedures). However, the extraction efficiency of linoleic acid increased with methanol concentration. For ultrasonic extraction, 100 % methanol gave the highest extraction efficiency of linoleic acid (around 0.8 mg/g). To verify the effect of extraction solvent volume, samples were extracted using different volumes of methanol (5, 10 and 20 v/w); however, increasing the solvent did not result in higher yields, where 5:1 v/w ratio was the most efficient. After that, the effect of extraction time was investigated by using times of 30, 60, 90 and 120 min. The extraction efficiency of linoleic acid increased with extraction time; however, the yield of linoleic acid did not change after 2 hours of sonication. Thus, the optimal extraction conditions, i.e. those that resulted

Table 3. Content of linoleic acid in reference AR and 12 samples purchased in China and Korea and evaluation of the samples

Sample No.	Linoleic acid (%)	RSD (%)	Species	Evaluation	Production
Reference	0.025	9.0	Adenophorae Radix	authentic	MFDS
S1	0.052	10.5	Adenophorae Radix	authentic	China
S2	0.007	12.1	*C. lanceolata*	substitute	Korea
S3	0.040	12.1	Adenophorae Radix	authentic	China
S4	0.005	12.5	*C. lanceolata*	substitute	Korea
S5	0.071	7.6	Adenophorae Radix	authentic	China
S6	0.005	14.3	*C. lanceolata*	substitute	Korea
S7	0.058	3.0	Adenophorae Radix	authentic	China
S8	0.006	10.2	*C. lanceolata*	substitute	Korea
S9	0.015	7.1	*C. lanceolata*	substitute	Korea
S10	0.012	10.0	*C. lanceolata*	substitute	Korea
S11	0.005	14.4	*C. lanceolata*	substitute	Korea
S12	0.004	14.2	*C. lanceolata*	substitute	Korea

in the highest yields of linoleic acid for 1.0 g of sample, were sonication for 90 minutes with 100 % methanol.

3.5. Discrimination of authentic AR and substitutes

The linoleic acid content, in reference AR and the 12 samples, was calculated using a calibration curve. As shown in *Table* 3, authentic AR samples demonstrated higher concentrations of linoleic acid than the Korean substitute samples; the linoleic acid content in authentic samples ranged from 0.040~0.071 % (mean = 0.056 %), while those in substitute samples ranged from 0.004 %~0.014 % (mean = 0.007 %). In comparison, the reference AR contained 0.025 % linoleic acid, suggesting that the Chinese AR samples were authentic while the Korean samples were not. Discrimination of authentic AR from samples by means of the content of linoleic acid would not be sufficient because some inauthentic or substitute samples contain also small amount of linoleic acid. Hence, cluster analysis was achieved with three variables that were peak areas of unknown peaks at 5.1 min, 9.9 min and linoleic acid at 13.8 min. This analysis split the 13 samples (including the reference AR) into two clusters. Authentic samples (S1, S3, S5 and S7) and the AR standard belonged to cluster I, while the remaining samples (S2, S4, S6, S8, S9, S10, S11 and S12) belonged to cluster II (*Fig.* 4). This classification was confirmed by thin-layer chromatography (TLC) results, as well as through sensory evaluation by a herbal specialist (Dr. Bae Ki Hwan, Chungnam National University). Finally, we evaluated that the 8 Korean samples were the root of *Codonopsis lanceolata* Trautv, a commonly used herbal proxy for AR. The linoleic acid content of the authentic samples was over 0.040 %, while that of the reference AR was 0.025 %. We therefore suggest a linoleic acid content criterion for AR of 0.02 %. Using this content criterion, we thus conclude that the Korean samples were all substitutes. This indicates that our suggested linoleic acid content criterion can be used to discriminate between AR and substitutes.

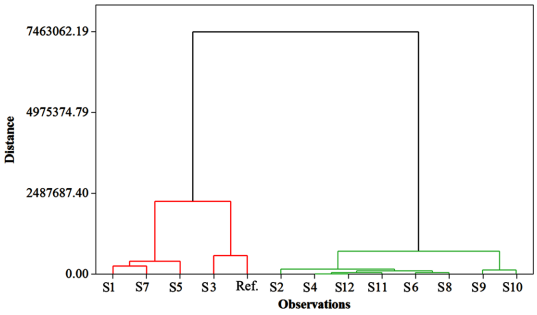

Fig. 4. Discrimination of authentic and substitute Adenophorae Radix by HCluster using three variables.

4. Conclusions

We developed a simple and fast HPLC-UV method

to identify authentic and inauthentic AR according to linoleic acid content. The method was well-validated with respect to linearity, precision, accuracy, and repeatability. Collected samples were classified as authentic or substitutes using the suggested linoleic acid content criterion (0.20 % of linoleic acid) and HCluster analysis of HPLC results. Our HPLC-UV method is thus suitable for identifying authentic AR quickly and accurately in commercial herbal products.

Acknowledgements

This work was supported by the research funds from Chungnam National University (Daejeon, Republic of Korea).

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