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Separation of liquiritin, glycyrrhizic acid and glabridin from licorice by RP-HPLC

Minglei Tian, Hongyuan Yan and Kyung Ho Row★

Center for Advanced Bioseperation Technology,
Department of Chemical Engineering, Inha University, Incheon 402-751, Korea
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RP-HPLC를 이용한 감초에서 liquiritin, glycyrrhizic acid, glabridin의 분리

전명래 · 염홍원 · 노경호★

초정밀생물분리기술연구센터, 화공과, 인하대학교
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Abstract: Reversed-phase high performance liquid chromatography (RP-HPLC) was used for the simultaneous determination of liquiritin (LQ), glycyrrhizic acid (GA) and glabridin from licorice. An optimized run condition was selected with a binary gradient elution of methanol-water which ramped 35/65 to 80/20 (vol. %) in 0.0-8.0 min and a flow rate of 1.0 mL/min. A good linearity was obtained between 0.2 mg/mL and 1.0 mg/mL for LQ and GA, and 0.01 mg/mL-0.2 mg/mL for glabridin with the relative standard deviations less than 0.90% (n=5). The developed method was successfully applied to determination of the three components from licorice samples. The mean recoveries of three components are 80.79% for liquiritin, 89.71% for glycyrrhizic acid and 72.50% for glabridin.

요 약: 역상 고성능 액상 크로마토그래피(RP-HPLC)를 사용하여 감초에 포함된 liquiritin (LQ), glycyrrhizic acid (GA)과 glabridin을 분석하였다. 이동상의 조성에 대한 실험을 수행한 실험결과에 의하면, 최적화된 이동상은 메탄올-물 이성분계이다. 이동상의 유량은 1 mL/min으로 일정하였고, 초기 8분동안에 35/65 (MeOH/water, v/v%)에서 80/20으로 구배용매조성법을 적용하였고, 그 이후에는 80/20으로 유지하였다. LQ과 GA의 농도는 0.2 mg/mL에서 1.0 mg/mL까지, glabridin의 농도는 0.01 mg/mL에서 0.2 mg/mL의 범위에서 선형성을 보였고, 상대 표준편차는 0.90%(n=5) 보다 낮았다. 개발된 방법을 사용하여 감초에 포함된 위의 세가지 성분의 정량분석을 성공적으로 수행하였다. 평균 수율은 LQ가 80.79%, GA가 89.71%, glabridin이 72.50%이었다.

Key words : liquiritin, glycyrrhizic acid, glabridin, licorice, RP-HPLC

★ Corresponding author
Phone : +82-(0)32-860-7470 Fax : +82+(0)32-872-0959
E-mail: rowkho@inha.ac.kr

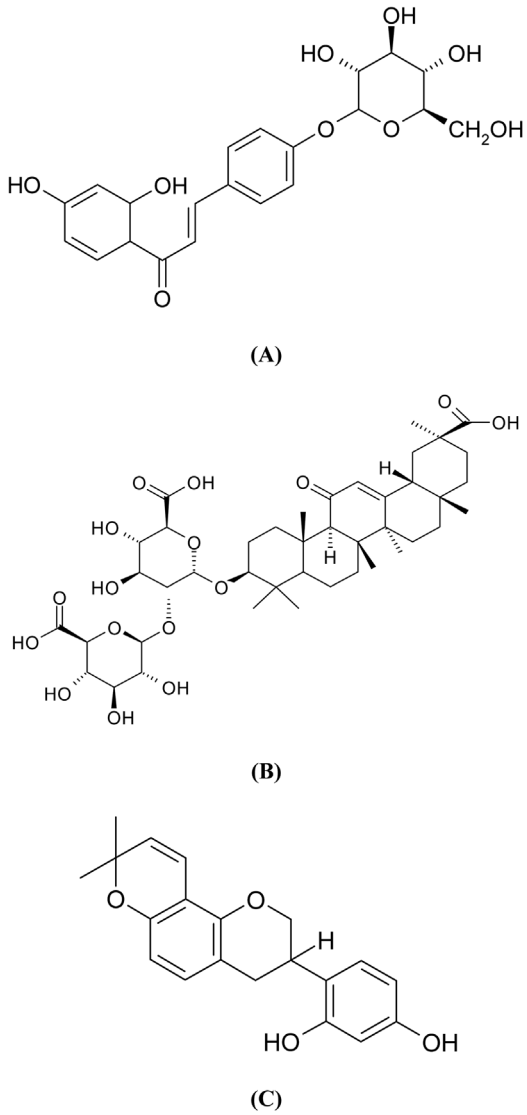

Fig. 1. Molecular structures of liquiritin (A), glycyrrhizic acid (B) and glabridin (C).

1. Introduction

Licorice, the root of the *glycyrrhiza* plant species, has been used as medicines more than 4000 years.[1] It is a Chinese herb commonly used as an expectorant, reduce fever, arrest coughing, comfort the stomach, alleviate urgency and potentiate the effect of various other herbs.[2] Liquiritin (LQ) (*Fig*. 1 (A)) is one of the most prevalent flavonoids in licorice[3] which has antioxidative[4] and anti-viral[5] properties.[6]

The most studied active constituent in licorice is a sweet-tasting material, glycyrrhizic acid (GA) (*Fig*. 1 (B)), which is 50 times sweeter than sugar and is widely used as a sweetening additive in the food industry.[7] In many countries, GA is used as a major therapeutic agent to treat chronic viral hepatitis and allergic dermatitis.[8] It also has anti-inflammation,[9] anti-ulcer,[10] anti-hepatotoxic and antivirus activities.[11,12] Glabridin (*Fig*. 1 (C)) is another active component in licorice. It exhibits multiple pharmacological activities, such as antimicrobial activity, cytotoxic activity, estrogenic and anti-proliferative activity against human breast cancer cells. It also affects on low-density lipoprotein oxidation, melanogenesis, inflammation, and protects of mitochondrial functions from oxidative stresses.[13]

There have been few reports on the separation of LQ, GA and glabridin, respectively, such as S. Shen, *et al.*[14] and C. Sun, *et al.*[15] However, method for the simultaneous separation of these three compounds was still not established. So the purpose of this study is to develop a new method to separate LQ, GA and glabridin from licorice simultaneously by RP-HPLC. Compared with the previously results, the present method is simple, rapid and could be used to simultaneously separate the three compounds simultanearsly from licorice.

2. Experimental

2.1. Chemicals

Liquiritin was obtained from National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). Glycyrrhizic acid (mono-ammonium salt hydrate) was purchased from Sigma Chemical Co. (St. Louis, MO) and glabridin was from Wako Pure Chemical Industries, Ltd. (Japan). Licorice was purchased from local market. Methanol (HPLC Grade) was obtained from Duksan Pure Chemical. Co., Ltd. (Korea) and trifluoroacetic acid (TFA) was from Acros Organics (USA). Water was twice distilled and filtered (FH-0.45 μm, Advantec MFS, Inc., Japan) by using a decompressing pump (Division of Millipore, Waters, USA).

2.2. HPLC analysis

The HPLC system in this study is comprised of a M930 solvent delivery pump (Young Lin Co. Korea), an UV detector (M 720 Absorbance Detector, Young-In Scientific Co., Korea) and an integrated data system (Autochrowin. Ver. 1.42, Young Lin Co., Korea). The HPLC column (C_{18}, 5 μm, 150×4.6 mm) was purchased from RStech Corporation (Daejeon, Korea) and a Reodyne injection valve with a 25 μL sample loop was used. The injection volume was 10 μL, the flow rate was 1.0 mL/min and UV wavelength was set at 252 nm

2.3. Sample preparation

The standards of LQ, GA and glabridin were dissolved in methanol to yield a final concentration of 0.33 mg/mL. 1.0 g licorice sample was dipping with methanol for 4 hours and the upper extracts were filtered by Disposable Syringe Filter Unit (0.2 ìm) for further HPLC analysis. All experiments were carried out at ambient room temperature.

2.4. Method validation

The retention factor (k) of these compounds was calculated as follows:

$$k = \frac{t_R - t_0}{t_0} \quad (1)$$

And the resolution (R) of these compounds was calculated as follows:

$$R = \frac{t_{R2} - t_{R1}}{\frac{w_1 + w_2}{2}} \quad (2)$$

Where t_R and t_0 are the retention times of analyte and unretained solutes, respectively, w_1 and w_2 are the baseline widths of the peaks.

The peak area values were plotted against the corresponding concentrations of the analytes and the calibration curves constructed by the means of least-square method. 0.2, 0.4, 0.5, 0.8, and 1.0 mg/mL of LQ, GA standards solutions and 0.01, 0.04, 0.08, 0.1, and 0.2 mg/mL of glabridin were applied, each concentration was injected 5 times.

The repeatability calculated as relative standard deviations (RSDs) were performed by injecting the standard solutions of LQ, GA and glabridin 5 times in one day and in 5 different days. The concentrations of standard solutions were 0.33 mg/mL and 10 μL was used as the injection volume.

Three concentrations of LQ (0.15, 0.20, 0.30 mg/mL), GA (0.5, 0.6, 0.8 mg/mL), and glabridin (0.05, 0.06, 0.07 mg/mL) were added to 3 ml of real sample to a final volume of 6 mL. Each sample was injected 3 times. The measured concentration was compared with the theoretical concentration to calculate the recoveries. The limit of detections were calculated according to Y. Lee, *et al* .[16]

3. Results and Discussion

3.1. Effect of mobile phase compositions

Isocratic elution was first investigated by changing the compositions of methanol-water from 80/20 to 0/100 (vol. %). From the results in *Table* 1, glabridin were easily eluted out with methanol-water (80/20, vol. %) as the mobile phase but LQ and GA were eluted as the same peak. With the compound of

Table 1. Retention factors of LQ, GA and glabridin with different mobile phases

Mobil phase \ k Compounds	LQ	GA	glabridin
Methanol/water (80/20, vol. %)	0.16	0.17	2.43
Methanol/water (70/30, vol. %)	0.35	0.59	7.21
Methanol/water (50/50, vol. %)	0.66	2.62	k <6.52*
Methanol/water (30/70, vol. %)	3.68	k <14.04 *	k <14.04*
Methanol/water (20/80, vol. %)	12.95	k <21.56 *	k <21.56*
Methanol/water (0/100, vol. %)	k <14.04 *	k <14.04 *	k <14.04*

*: no peak before

Table 2. Experimental conditions and retention coefficients

Run No.	Initial composition (methanol/water, vol. %)	First gradient time (min)	Second composition (methanol/water, vol. %)	Second gradient time (min)	Final composition (methanol/water, vol. %)	k			R	
						LQ	GA	glabridin	R_{LQ-GA}	$R_{GA-glabridin}$
1	25/75	8	25/75	12	70/30	7.20	16.79	27.75	10.04	13.62
2	25/75	7	25/75	9	70/30	7.20	14.40	25.04	8.63	16.36
3	25/75	7	25/75	9	90/10	6.89	13.43	16.52	7.82	7.62
4	30/70, +0.05 vol. % TFA	7	30/70, +0.05 vol. % TFA	9	80/20,+0.05 vol. % TFA	9.48	17.66	18.70	9.10	2.08
5	25/75, +0.1 vol. % TFA	7	25/75, +0.1 vol. % TFA	9	70/30, +0.1 vol. % TFA	12.83	17.91	25.05	9.08	10.98
6	30/70, +0.1 vol. % TFA	7	30/70, +0.1 vol. % TFA	9	80/20, +0.1 vol. % TFA	9.49	17.68	18.71	9.11	2.06
7	35/65, +0.1 vol. % TFA	7	35/65, +0.1 vol. % TFA	9	80/20, +0.1 vol. % TFA	5.16	17.38	18.39	16.09	1.92
8	35/65, +0.1 vol. % TFA	5	35/65, +0.1 vol. % TFA	3	90/10, +0.1 vol. % TFA	4.86	11.53	11.53	9.01	1.83
9	35/65	Gradient time (min) 8			80/20	2.22	6.54	11.43	5.08	3.32

methanol decreasing in water, LQ and GA could be separated well but glabridin was not eluted out less than 30 min. In this case the gradient elution mode was applied for further work.

Firstly, the concentration of methanol in the mobile phase was increased from 25% to 75% when gradient profile from 9 min to 12 min. All the three compounds could be separated and the total running time less than 40 min. As seen from peak No.1 and peak No. 2, the retention factors of GA and glabridin decreased with the gradient time decreasing. So shorter gradient time was used for next experiment.

Then the initial compositions of methanol-water increased from 25/75 to 35/65 (vol. %). From the results in *Table* 1, the retention factors of LQ decreased with the compositions increasing, and it was eluted out less than 5 min. So methanol-water (35/65, vol. %) was chose as the optimum initial composition. Afterward, the final compositions of methanol-water increased from 70/30 to 90/10 (vol. %). The results in *Table* 2 indicated that the retention factors and resolutions of GA and glabridin decreased with the compositions of methanol-water increasing. According to the value of resolution (*R*) the composition of methanol-water (90/10, vol. %) was used and the result was shown in *Fig*. 2.

3.2. Effect of mobile phase additives

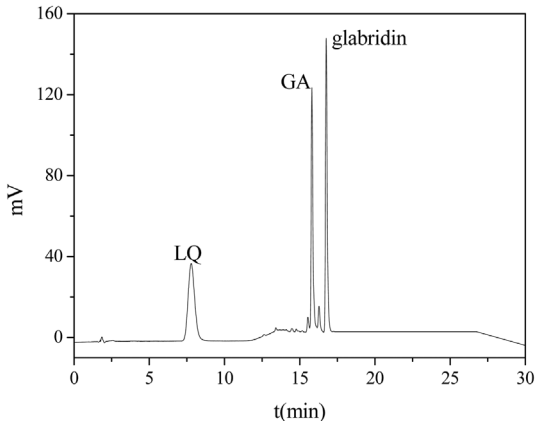

Fig. 2. Separation of LQ, GA and glabridin by RP-HPLC (# 8 in *Table* 2, 10 mL injection, 0.33 mg/mL, 1.0 mL/min)

In order to determine the effect of trifluoroacetic acid (TFA) as modifier, 0.05-0.1% TFA in mobile phase was investigated. Compared the results of No.2 with No.5 and No. 4 with No. 6 in *Table* 2, the retention factors of LQ, GA obviously increased after using the TFA but the retention factor of glabridin was not changer obviously, so 0.1% TFA was obtained.

3.3. Validate the separation conditions of real sample

The chromatogram of the extract from licorice was shown in *Fig*. 3, the peaks of GA and glabridin were conflicted and could not be separated. So the

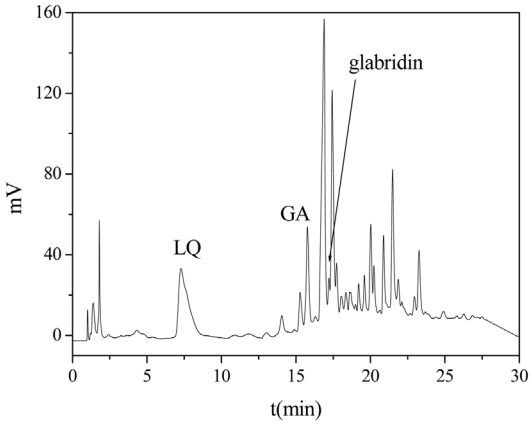

Fig. 3 Chromatogram of the extracts of licorice (# 8 in *Table* 2, 10 mL injection, 1.0 mL/min).

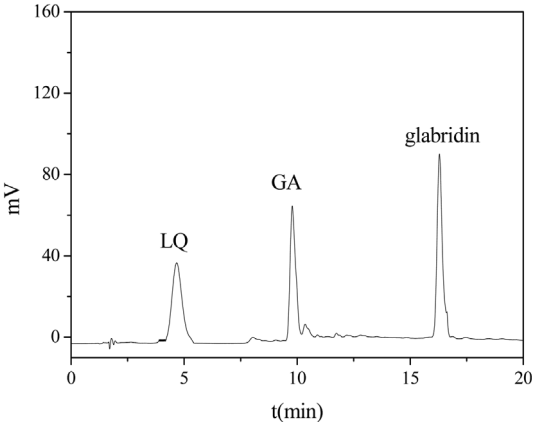

Fig. 4. Separation of LQ, GA and glabridin by RP-HPLC (# 9 in *Table* 2, 10 mL injection, 0.33 mg/mL, 1.0 mL/min).

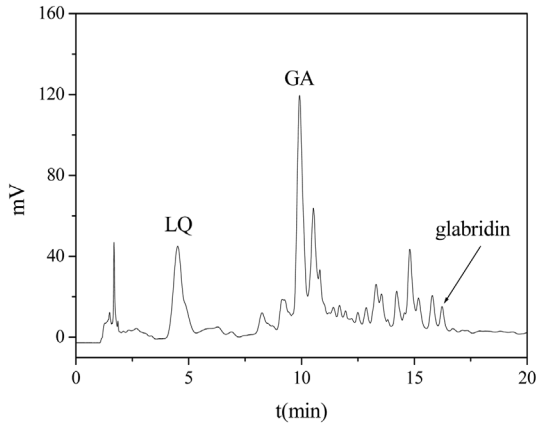

Fig. 5. Chromatogram of the extracts of licorice (# 9 in *Table* 2, 10 mL injection, 1.0 mL/min).

Table 3. Extracted amounts of LQ, GA and glabridin from licorice

Compound (mg/g) No.	LQ	GA	glabridin
Sample # 1	0.21	1.43	0.070
Sample # 2	0.19	1.39	0.065
Sample # 3	0.20	1.40	0.073

separation condition should be further modified according to the real sample.

The modified gradient mode was listed in No. 9 of *Table* 2 and the chromatogram was shown in *Fig*. 4. By this way, the total run time was nearly same as No. 8 but the three components from licorice were separated well. *Fig*. 5 shows the chromatogram of licorice extracts and *Table* 3 shows the extraction amounts of the three compounds from licorice.

3.4. Method validation

The regression equations of LQ, GA, and glabridin were y=8414.2x-224.54, y=3727.7x-51.498 and y=5323.3x-29.155, respectively, where y is the area of analyte peak, x is the concentration expressed as 0.2-1.0 mg/mL for LQ and GA, 0.01-0.2 mg/mL for glabridin, the linear correlation coefficients (r^2) were 0.9999 for LQ, 0.9996 for GA and 0.9997 for glabridin. The RSD of precision tests, the limit of detections (LOD), recoveries were in *Table* 4. Comparison with the real sample analysis verified that the values noted above were of acceptable precision and accuracy.

4. Conclusion

In this experiment, a convenient method was developed for the simultaneous separation of LQ, GA and glabridin from licorice. Effect of the compositions of mobile phases, gradient profile and the concentrations of mobile phase additive were evaluated. The developed method was successfully validated and applied to determine the three components in Chinese licorice sample.

Acknowledgements

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Table 4. RSDs, Recovery and LODs of the three compounds from licorice

Compounds	Added concentrations (mg/mL)	Precision		Recovery		LOD (ng/mL)
		Intra-day (%)	Inter-day (%)	Recovery (%)	RSD (%)	
LQ	0.15	0.34	0.37	81.3	0.26	365
	0.20			79.9	0.29	
	0.30			81.0	0.28	
GA	0.5	0.54	0.59	88.7	0.46	464
	0.6			90.1	0.45	
	0.8			90.3	0.47	
Glabridin	0.05	0.83	0.90	74.5	0.79	229
	0.06			69.9	0.76	
	0.07			73.2	0.78	

Technology and Inha University, Korea.

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