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Preparative Purification of the Major Flavonoid Glabridin from Licorice Roots by Solid Phase Extraction and Preparative High Performance Liquid Chromatography

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This study investigated a novel, simple, and economical method for the preparation and purification of glabridin from licorice roots. Glabridin was initially obtained from ethyl acetate extraction of licorice, followed by using solid phase extraction (SPE) and preparative high performance liquid chromatography (HPLC). The content of glabridin increased from 0.23% to 35.2% after SPE, and then a 16 mg product at a high purity of over 95% was obtained from 10 g licorice roots after purification by preparative HPLC. The purity was assessed by analytical HPLC, and the purified compound was characterized by LC-MS/MS and ^{1}H NMR.

Keywords glabridin; licorice; preparative high performance liquid chromatography; purification; solid phase extraction

INTRODUCTION

The root of licorice (*Glycyrrhiza glabra* L.) is a traditional medicinal herb of *Leguminosae* family and has been used medically for more than 5000 years (1). It is used frequently to treat various diseases in respiratory, digestive, endocrine, cardiovascular, and the central nervous system in traditional Chinese medicine (2,3). Licorice roots contain many bioactive components, such as glycyrrhizin, liquiritin, liquiritenin, and glabridin. Among these functional components, glabridin, as the major polyphenolic flavonoid, is found in the cork layer and decayed part of its thickening roots (4). Glabridin has revealed a wide variety of pharmacological activities, such as cytotoxic activity (5) and antimicrobial activities against *Helicobacter pylori* (6). And it also exhibited significant activities including estrogenic and anti-proliferative activity against human breast cancer cells (7), anti-inflammatory and skin whitening effects (melanogenesis inhibiting) (8), anti-oxidative effects of low density lipoprotein (LDL) (9), and protection of mitochondrial functions from oxidative stresses (10).

As a kind of estrogenic, antioxidant, and powerful skin whitening ingredient, glabridin has been widely used in pharmaceutical, food, and cosmetic industries. The fact is that the glabridin extraction and purification technologies have drawn increasing attention from various groups of people. The conventional separation process of glabridin was achieved through solid-liquid extraction from natural resources, and then liquid-liquid extraction was conducted by using different solvents, and lastly, by using gel chromatography for several times (11). However, only a limited amount of purity of glabridin was obtained by applying this time-consuming method, which also requires large amount of solvents.

Recently, several more efficient methods were developed to improve the process of separation of glabridin from licorice roots, such as supercritical fluid extraction (12), macroporous resins separation (13,14), and a selective molecularly imprinted solid-phase extraction procedure (15). These methods are more effective and rapid than the conventional method. In addition, some drawbacks must be taken into consideration, these methods are inefficient regarding reagents, low recoveries of the product, and the purity of glabridin is not high enough. In recent studies, even though preparative HPLC is a valuable method for covering the demand of glabridin, the existing preparative HPLC method for the purification of glabridin (16) is not well suited for large-scale productions. As a result, it is important to find a possible, effective, and cost-effective method for the preparation of high purity glabridin.

Nowadays, the combination of SPE and preparative HPLC is used to meet the specified purity requirements on a routine basis, and it is also an important method of applied industrial separation for the isolation and purification of valuable products (17–19). However, no studies have involved the combined method of SPE and preparative HPLC for isolation and purification of glabridin from licorice roots.

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In this study, a simple and highly efficient method was established for the separation and purification of glabridin using SPE and preparative HPLC based on a method developed in our laboratory for the preparation of glabridin from licorice roots.

EXPERIMENTAL

Chemicals and Reagents

The roots of licorice were purchased in China National Group Corporation of Traditional & Herbal Medicine (Beijing, China). Glabridin standard was purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Hexane and ethyl acetate were of analytical grade and purchased from Beijing Chemical Factory (Beijing, China). Trifluoracetic acid (TFA) used for HPLC analysis was of chromatography grade and purchased from Merck Co. (Hohenbrunn, Germany). Acetonitrile and methanol were of chromatography grade and purchased from Dima Technology Inc. (California, USA). Silica gel (200–300 mesh, irregular) was obtained from the Haiyang Chemical Group (Qingdao, China).

Preparation of Crude Licorice Extracts

Fifty grams of licorice roots (*Glycyrrhiza glabra L.*) was grounded into powder, and then 10 g of dried powder was extracted by sonication with 80 mL ethyl acetate for 30 min. After filtration, the filtrate was collected and the solid was extracted again (two times) with the same volume of fresh solvent. The combined extraction solution was evaporated to dryness at 40°C.

HPLC Analysis of Glabridin

The HPLC we used was a Shimadzu LC-20AVP system with two LC-20AT solvent delivery units, an SPD-20A UV/VIS detector, a CTO-10ASVP column oven (Shimadzu, Kyoto, Japan), T2000P workstation (Beijing, China) and a reversed phase C_{18} column (250×4.6 mm, 5 μm, Diamodsil^{TM}). Conditions for HPLC detection of glabridin are as follows: acetonitrile (eluent A) and 0.05% (v/v) TFA in water (eluent B). The elution started with 60% acetonitrile and lasted for 20 min, and then acetonitrile concentration was raised to 85% during 2 min and maintained for 15 min to purge the column (14). The column oven temperature was set at 30°C. The flow rate was 1.0 mL/min, 10 μL samples were injected into the column and detected by UV at 283 nm.

Solid Phase Extraction

SPE of glabridin from the crude extracts was performed in a glass column (12 mm × 50 mm) packed with silica gel. The crude extract was dissolved in 4 mL of ethyl acetate-hexane (1:4, v/v). The silica gel about 5 g (200–300 mesh) was activated for 60 min at 120°C, then it was mixed with 20 mL of hexane, and the slurry was packed into the SPE column. The SPE column was conditioned with 20 mL of hexane and 4 mL of sample solution at the glabridin concentration of 3.68 mg/mL successively. The flow rate of the solvent and sample solutions through the column was controlled at 2–3 mL/min under vacuum. The column was sequentially rinsed with 50 mL of ethyl acetate-hexane (1:4, 1:2, 1:1, v/v, respectively) solutions. Each part of the eluate was evaporated at 35°C under vacuum by a rotary evaporator, and was dissolved in 30 mL methanol by sonication for approximately 1 min, then was filtered through the 0.22 μm membrane, and was analyzed by HPLC before subjection to preparative HPLC.

Preparative HPLC Separation

Reversed-phase preparative HPLC was carried out on a Waters Prep 4000 liquid chromatography system equipped with a fluid handling unit (pump heads), controller (for solvent gradient, flow rate, external events, and sparging process) and a 2487 dual-wavelength absorbance detector with a preparative detection cell (Waters, Milford, USA). The chromatographic separation was performed on a Symmetry C_{18} column (300×19 mm, 15 μm). Detection was carried out by UV at 283 nm.

Preparative HPLC separation was performed by injecting 2 mL of glabridin-rich extracts. Elution was performed using Waters model 501 pump to deliver a constant flow rate of 10 mL/min. The solvent system consisted of 70% (v/v) methanol in water for 40 min, then the percentage of methanol was raised to 100% (v/v) over 2 min and run isocratically for 20 min to purge the column. Glabridin was collected between 35 and 37 min, and was analyzed by HPLC. The solvent was dried at 40°C under vacuum on a rotary evaporator to obtain purified compound.

Validation of Glabridin

The preparative monomer of glabridin was identified by LC-MS/MS and ^{1}H NMR. Glabridin was injected into C_{18} column (250×4.6 mm, 5 μm, Diamodsil^{TM}), using a Surveyor Autosampler (Thermo Finnigan, USA). The column was maintained at 30°C with elution of 70% mobile phase A and 30% mobile phase B at a flow rate of 0.4 mL/min. Solvents A, B were acetonitrile and 2% formic acid, respectively. Full MS scan was operated in positive and negative modes using a Thermo Finnigan LCQ Advantage ion trap mass spectrometer equipped with electrospray ionization (ESI) source. The ESI-MS instrument was operated with the capillary temperature at 250°C, sheath gas at 0.80 L/min and the auxiliary gas at 0.10 L/min. The electrospray voltage was set to 4 kV, the capillary voltage at 32 V and the tube lens offset at −5 V. The selected reaction of glabridin was monitored with relative collision energy of 30%.

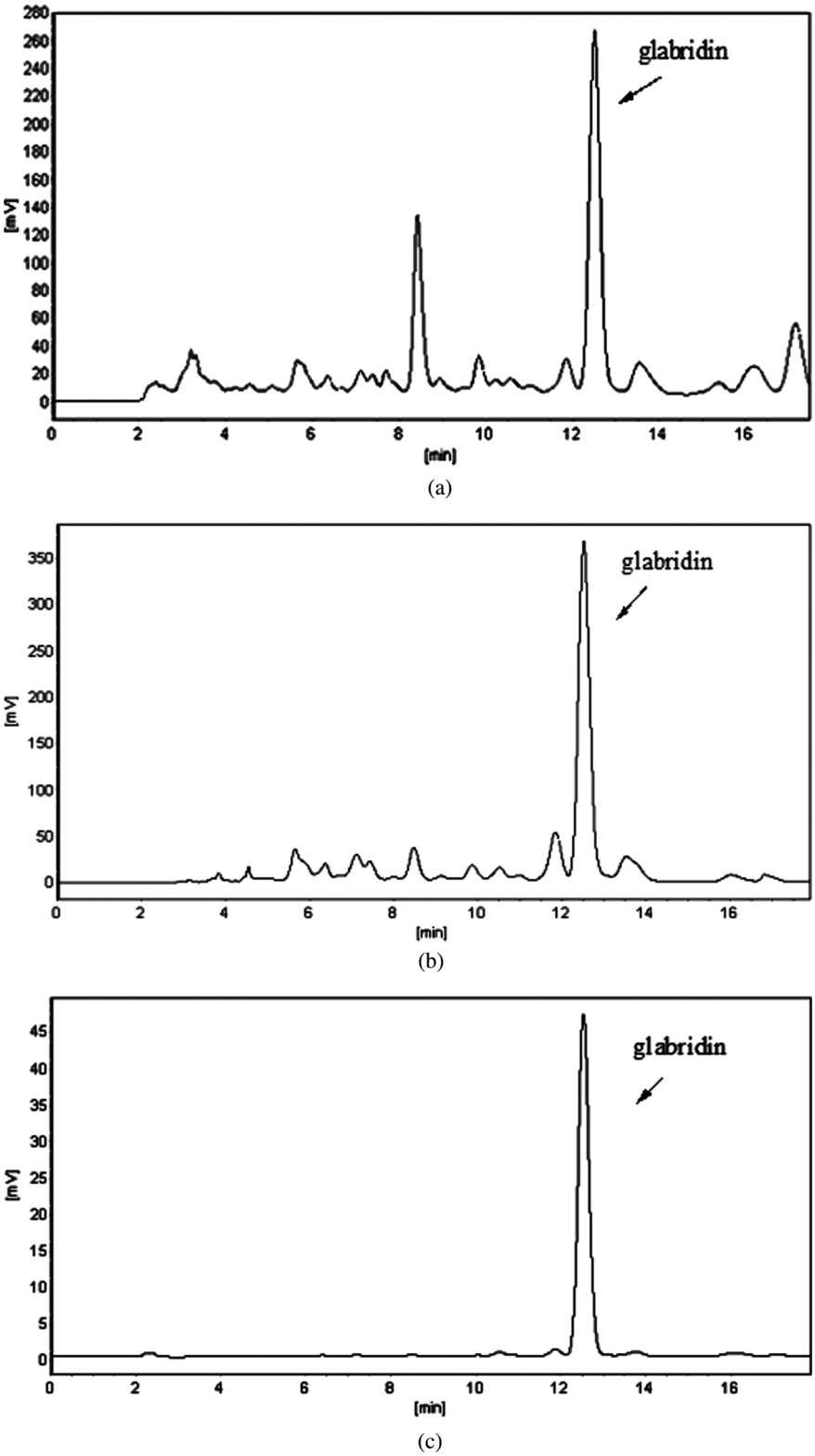

FIG. 1. Analytical HPLC chromatograms of glabridin obtained by crude extraction (a), SPE (b), and preparative HPLC (c). Conditions: column: reversed phase C_{18} column (250×4.6 mm, 5 μm, Diamodsil^{TM}); mobile phase: acetonitrile and 0.05% (v/v) TFA in water, the eluation started of 60% acetonitrile and lasted for 20 min, then acetonitrile was raised to 85% during 2 min and maintained for 15 min to purge the column; flow rate: 1.0 mL/min; detection wavelength: 283 nm; column oven temperature: 30°C.

^{1}H NMR spectra were performed in CD_3COCD_3 using a Bruker high-resolution AV600NMR spectrometer at 600 MHz (Bruker Biospin Corporation, Billerica, MA).

RESULTS AND DISCUSSION

Quantification of Glabridin by Analytical HPLC

The concentration of glabridin was analyzed by HPLC. A good separation effect for glabridin was obtained by implementing our methodology, and the retention time for glabridin was 12.5 min (Fig. 1).

The linear regression curve was obtained by plotting the peak area responses (y) versus the theoretical concentration (x, mg/mL). The regression equation was given: $y = 1.10 \times 10^7\, x + 2.86 \times 10^4$, correlation coefficient (r^2) = 0.9999. The calibration curve was prepared in range from 0.0125 to 2 mg/mL, which is satisfactory with regard to the actual content of glabridin.

The correlation coefficient demonstrated the linearity of the method over the analyzed concentration range. The system precision was determined by assaying 6 injections of the standard solution and calculating the relative standard deviation (RSD) of the peak area responses. The method precision was established by assaying 6 different extracts of the same sample with the proposed chromatographic method. The RSD% for standard and samples were 0.34 and 0.63, respectively.

Solid Phase Extraction

SPE is one of the most important preconcentration methods because of its simplicity, flexibility to choose the solid phase, high preconcentration factor, low consumption of the organic solvents, low cost, and short extraction time for sample preparation (20). The SPE procedure described here allowed for the removal of pigments that might potentially interfere with the separation or block the preparative HPLC column.

In order to reduce the consumption of reagents and make the purification effective, SPE was conducted as described. The mean content of glabridin determined by HPLC was 0.23% in the licorice and 6.23% in the extracts, respectively. Ethyl acetate-hexane (1:4 v/v) was added to obtain a sample solution containing glabridin in the concentration of 3.68 mg/mL. The results of the content, purity, and recovery of glabridin at different eluate solutions were summarized in Table 1. The following equation was used to quantify the recovery yield of glabridin:

$$Y = \frac{C_d V_d}{m} \times 100\% \qquad (1)$$

where Y is the recovery yield of glabridin (%); C_d is the concentration of glabridin in the elution solution (mg/mL); V_d is the volume of the elution solution (mL); m is the quality of the glabrindin sample (mg).

TABLE 1
Results of content, purity and recovery of glabridin at different eluate solutions of SPE

Eluate (ethyl acetate:hexane)	Content (mg)	Purity (%)	Recovery (%)
1:4	0.853	5.2	5.8
1:2	11.290	35.2	76.2
1:1	0.499	8.2	3.4

The results (see Table 1) indicated that the HPLC analysis in each part of eluate showed that most of glabridin were desorbed from the silica gel via ethyl acetate-hexane (1:2, v/v) solution. Compared with glabridin, the polarity of impurities was weaker or stronger so that these compounds were eluted with other eluates. Thus, silica gel was initially eluted with ethyl acetate-hexane (1:4, v/v) solution to remove impurities and subsequently eluted with ethyl acetate-hexane (1:2, v/v) solution to obtain the glabridin-rich extracts. The HPLC chromatogram of the glabridin-rich extracts obtained by SPE was shown in Fig. 1b. Results of HPLC analysis indicated that although many impurities of glabridin were removed by SPE, some other components, whose polarity is similar to glabridin, were also found in the glabridin-rich extract. These impurities should be further separated from the glabridin-rich fraction by preparative HPLC.

After removal of the solvent by a rotatory evaporator, 50 mg glabridin-rich extract was obtained by SPE from 10 g of crude licorice roots.

Solvent Selection in Preparative HPLC

For reversed-phase HPLC, water is used as the weak solvent against a stronger organic solvent which is used to elute compounds from the column. Acetonitrile and methanol are common solvents and powerful tools used to enhance selectivity during separation with an aqueous mobile phase in reversed-phase HPLC. Considering the principle of economy and toxicity, methanol has more advantages than acetonitrile. Furthermore, separation using methanol and water can achieve satisfactory result. Accordingly, methanol and water were selected as the basic mobile phase system for preparative HPLC.

Table 2 showed the effects of the variations in the mobile phase on the retention factor (k'), the selectivity factor (α) and the resolution factor (R_s) of glabridin for HPLC separation. Taking into account the requirement of scaling up, it is desirable to have the resolution factor (R_s) greater than 2, and the selectivity factor (k') less than 10 (18). In the research, we found that 70% methanol was the best

TABLE 2
Effects of the variations in the mobile phase on the retention time (t), the retention factor (k'), the selectivity factor (α) and the resolution factor (R_s) of glabridin for HPLC separation

Methanol concentration (%)	t	k'	α	R_s
60	78.184	31.509	1.185	3.399
65	40.875	15.996	1.154	2.930
70	23.338	8.721	1.124	2.058
80	9.618	2.999	1.149	1.598

mobile phase in the view of the separation effect and run time. Therefore, 70% (v/v) methanol in water was chosen as the mobile phase when considering the preparation of large amounts of samples.

Effects of Flow Rate and Sample Loading on Purification of Glabridin

The flow rate and sample loading may have a significant impact on the procedure of separation on preparative HPLC. In order to obtain optimum experimental conditions, the effects of flow rate and sample loading were studied and the results were presented in Tables 3 and 4. When one parameter was investigated, the other was kept constant. When the flow rate increased, the purity of glabridin increased at first and then decreased. It reached its peak value at a flow rate of 10 mL/min. When the sample loading increased, the purity of glabridin decreased. Consequently, in order to make the purification effective, the flow rate should be controlled at 10 mL/min and the loading amount should be limited to less than 20 mg. The purity of glabridin increased to over 95% after treatment by preparative HPLC. Finally, 70% (v/v) methanol in water as the mobile phase and a flow rate of 10 mL/min were applied to the separation of glabridin-rich extracts

TABLE 3
Results of retention time, purity and recovery of glabridin at different flow rates

Flow rate (mL/min)	Retention time (min)	Purity (%)	Recovery (%)
8	43	94.2	87.5
10	36	96.4	90.2
12	31	92.3	92.3
14	26	90.1	93.1

TABLE 4
Results of purity and recovery of glabridin at different sample loadings

Sample loading (mg)	Purity (%)	Recovery (%)
5	96.7	87.4
10	96.4	90.2
20	91.7	91.5
40	85.2	91.2

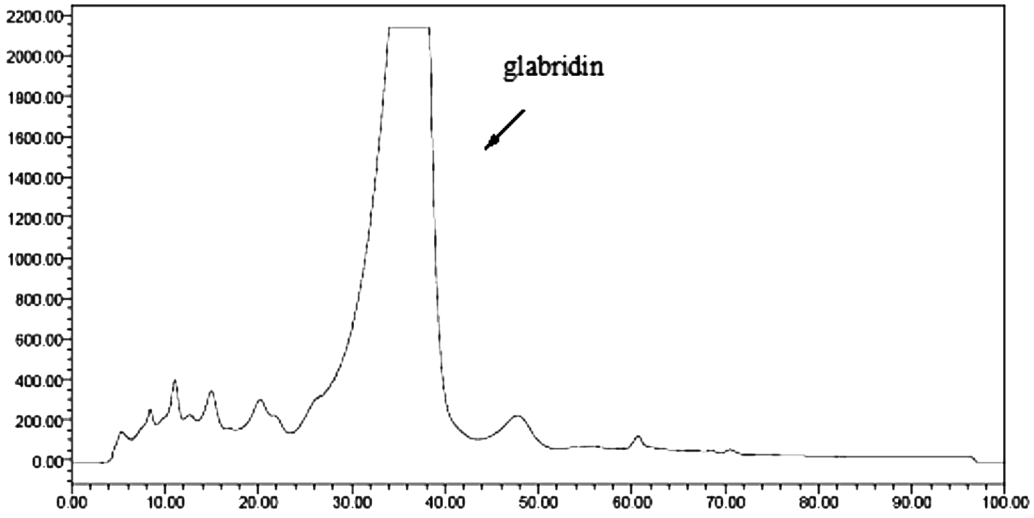

FIG. 2. Preparative HPLC chromatogram of glabridin extract. Conditions: column: reversed phase C_{18} (300 × 19 mm, 15 μm, Symmetry); mobile phase: 70% (v/v) methanol in water for 40 min, then the percentage of methanol was raised to 100% (v/v) over 2 min and run isocratically for 20 min to purge the column; flow rate: 10 mL/min; detection wavelength: 283 nm.

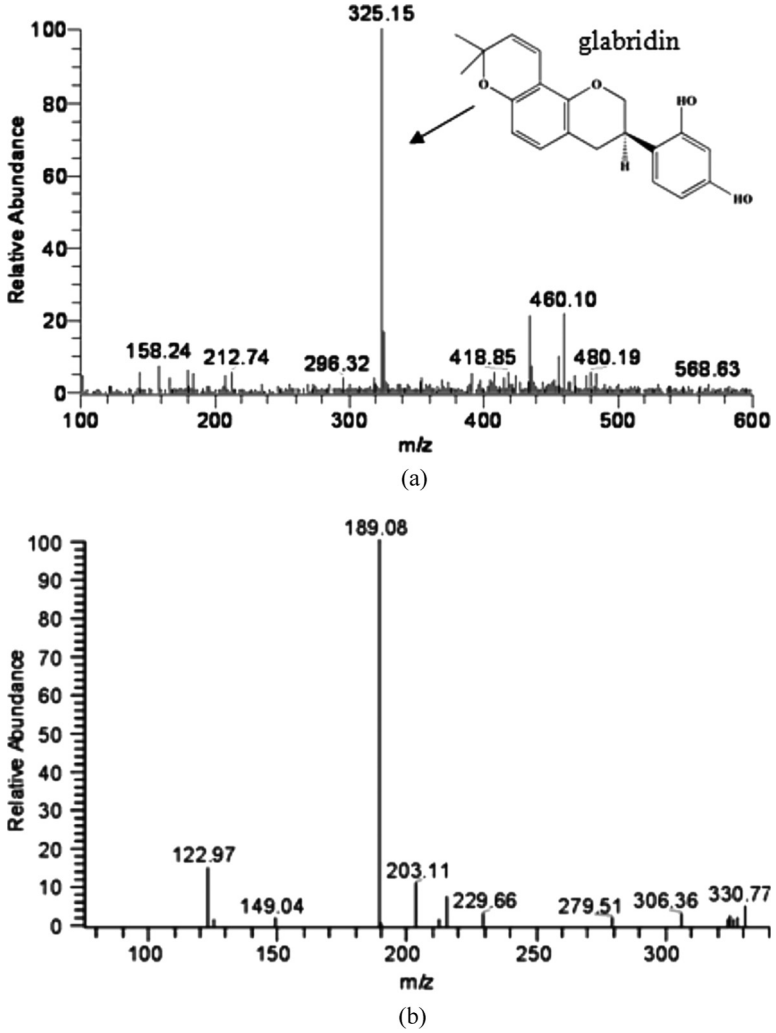

FIG. 3. LC-MS/MS of glabridin purified by preparative HPLC (a: MS, b: MS^2).

using preparative HPLC. Preparative HPLC chromatogram was shown in Fig. 2.

Chromatograms of glabridin obtained by crude extraction, SPE, and preparative HPLC were shown in Fig. 1. By comparison, most impurities were removed and the relative peak area of glabridin increased significantly after separation and purification. The content of glabridin increased from 0.23% to 35.2% after SPE and finally a 16 mg product at a high purity of over 95% was obtained from 10 g licorice roots after purification by preparative HPLC.

Confirmation of Glabridin

For further confirmation of the chemical structure of the product, purified samples were analyzed by LC-MS/MS. Figure 3 identified the MS and MS^2 mass spectra of the purified glabridin. The proton ion of glabridin (molecular weight 324.37) in full MS spectra was m/z 325 $(M+H)^+$ and the ion peak was detected in positive ion mode. While its main fragmentation ion in MS^2 spectra were m/z 123 (calculated for $C_8H_{11}O^+$), m/z 149 (calculated for $C_9H_9O_2^+$), m/z 189 (calculated for $C_{12}H_{13}O_2^+$) and m/z 203 (calculated for $C_{13}H_{15}O_2^+$), these fragment peaks were generated by collision energies, see Fig. 4. As can be seen, MS analysis of glabridin was consistent with the previously reported (1).

^{1}H NMR parameters were (600 MHz, acetone-D6, ppm): 1.37 and 1.38 (6 H, s, s, $(CH_3)_2$), 2.80 (1 H, m), 2.98 (2 H, m), 3.47 (1 H, ddd, $J = 14.89$, 10.65, 4.20 Hz), 4.01 (1 H, t, $J = 10.27$ Hz), 4.33 (1 H, m), 5.63 (1 H, d, $J = 9.92$ Hz), 6.28–6.97 (6 H, m), 8.52 (1 H, s). ^{1}H NMR analysis of the purified glabridin sample produced results that also were consistent with the previously reported (11), and did not show the presence of compounds other than glabridin.

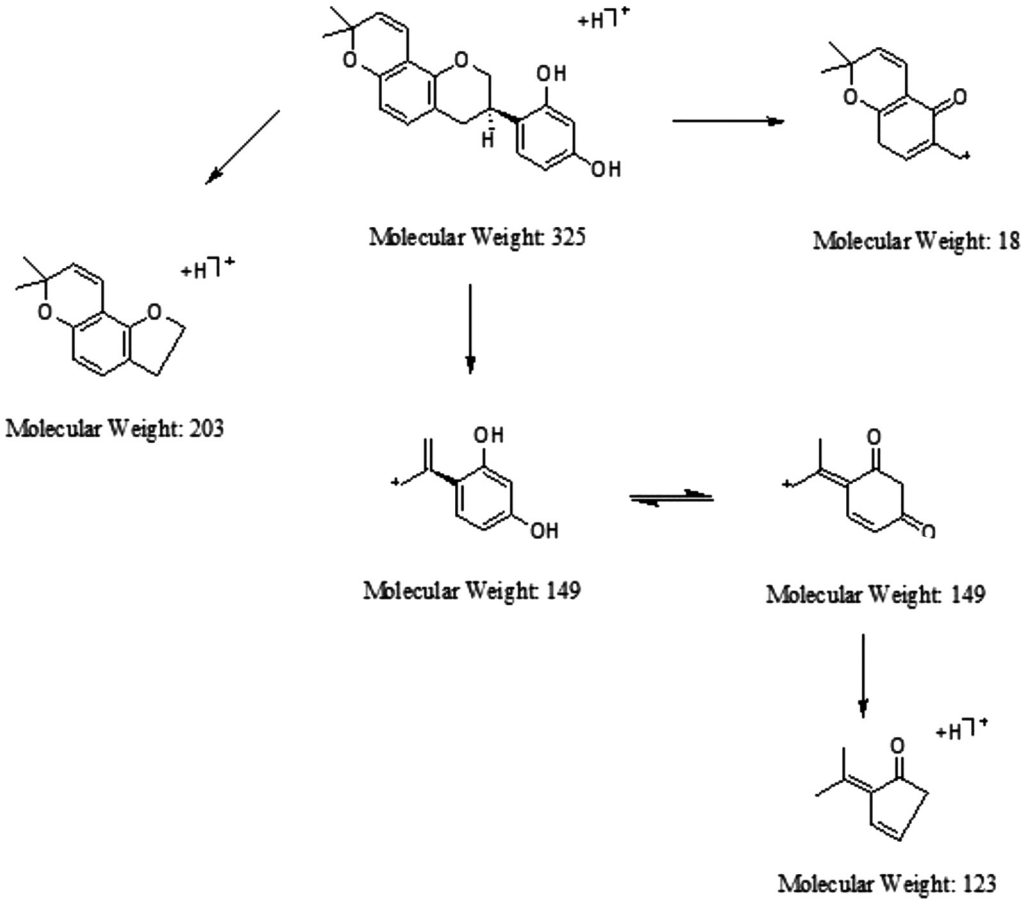

FIG. 4. ESI-MS^2 product ions obtained from glabridin.

CONCLUSIONS

This report presents a simple method for the preparation and purification of glabridin from licorice roots for the first time. The procedure included ethyl acetate extraction of licorice, SPE, and preparative HPLC. Glabridin was initially adsorbed in silica gel by SPE, then silica gel was eluted with ethyl acetate-hexane (1:4, v/v) solution to remove impurities and was subsequently eluted with ethyl acetate-hexane (1:2, v/v) solution to obtain the glabridin-rich extract. 70% (v/v) methanol in water was selected as the basic mobile phase system for preparative HPLC. The parameters of the flow rate and sample loading of preparative HPLC were optimized for the most effective purification. 16 mg glabridin at a high purity of over 95% was purified from 10 g licorice roots. The purity was assessed by analytical HPLC and the purified compound was characterized by LC-MS/MS and ^{1}H NMR. The method is simple, highly efficient, environmentally friendly, and has been demonstrated to be effective for the preparation and purification of glabridin from licorice roots.

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