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Determination of nine flavonoids and coumarins in licorice root by high-performance liquid chromatography

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ABSTRACT

A rapid high-performance liquid chromatographic method for the simultaneous separation and determination of five flavonoids and four coumarins, *viz.*, liquiritin, isoliquiritin, liquiritigenin, isoliquiritigenin, glycycoumarin, isoglycycoumarin, licochalcone A, glycyrol and isoglycyrol, in licorice root is described. The separation system consists of a reversed-phase column and a gradient elution system containing acetonitrile and 3% acetic acid in water. The compounds were detected at 310 and 365 nm successively. The recoveries of the flavonoids and coumarins were 95.6–105.2% with relative standard deviations of 0.62–4.24%. The contents of the above nine compounds in three species of licorice root produced in China were determined.

INTRODUCTION

Licorice root, the roots and rhizomes of *Glycyrrhiza* spp., family Leguminosae, has long been used as an important drug in China and also in Europe. It attracted special attention when Revers[1] in 1946 discovered and investigated the effect of licorice extract in the treatment of gastric and duodenal ulcers. Since then, a large amount of work on the chemical, pharmacological and clinical studies have been reported[2–5]. It has been found that the main active constituents of licorice root are saponins, flavonoids and coumarins, among which glycyrrhizinic acid and its aglycone showed anti-inflammatory, antitussive and antiallergic activities[3], liquiritin and isoliquiritin and their aglycones antiulcerogenic and spasmolytic activities[3,5], glycycoumarin antibacterial activity and licochalcone A anti-HIV activity[6].

Methods for the determination of glycyrrhizinic acid using precipitation[7], thin-layer chromatography[8], gas–liquid chromatography[9] and high-performance liquid chromatography (HPLC)[10–13] have been reported. No method for the determination of other constituents of licorice root has been found in literature. We describe here an HPLC method for the separation and determination of five flavonoids and four coumarins, *viz.*, liquiritin (**I**), isoliquiritin (**II**), liquiritigenin (**III**), isoliquiritigenin (**IV**), glycycoumarin (**V**), isoglycycoumarin (**VI**), licochalcone A (**VII**), glycyrol (**VIII**)

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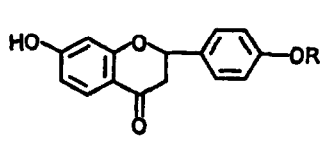
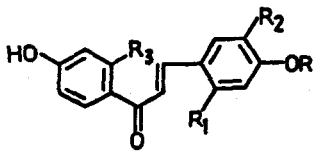
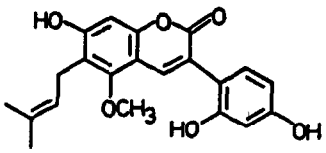


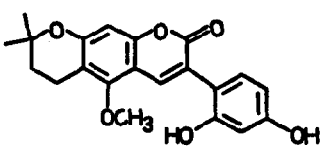


		R
I	Liquiritin	Glu
III	Liquiritigenin	H

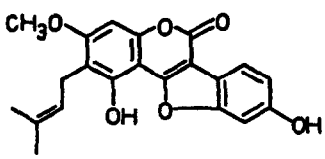
		R	R_1	R_2	R_3
II	Isoliquiritin	Glu	H	H	OH
IV	Isoliquiritigenin	H	H	H	OH
VII	Licochalcone A	H	OCH_3	$CMe_2CH{=}CH_2$	H



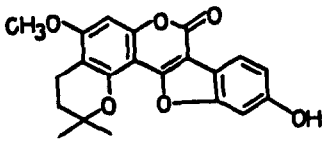
V Glycycoumarin



VI Isoglycycoumarin



VIII Glycyrol



IX Isoglycyrol

Fig. 1. Structures of compounds I–IX. Me = CH_3.

and isoglycyrol (**IX**). These compounds are known to be present in certain *Glycyrrhiza* spp.[4,16–18] and their structures are shown in Fig. 1.

EXPERIMENTAL

Plant material

Glycyrrhiza uralensis Fisch. was collected in Linxian County, Shanxi Province, and purchased from Nei Monggol Autonomous Region (July, 1987), *G. inflata* Bat. in Yianqi County, Xinjiang Autonomous Region (July 1986) and *G. glabra* L. in Hejing County, Xinjiang Autonomous Region (July, 1986). Roots and rhizomes of the above species were used in all analyses. Voucher specimens of the plants and crude drugs are deposited in the drug museum of the Department of Pharmacognosy, School of Pharmaceutical Sciences, Beijing Medical University.

Apparatus

HPLC analysis was carried out using a Varian 5500 instrument with a Varian UV-200 spectrophotometric detector, a Varian 4270 data processor and a Zorbax ODS stainless-steel column (15 cm × 4.6 mm I.D.) (Shimadzu, Kyoto, Japan).

Reagents

Freshly distilled acetonitrile and acetic acid of analytical-reagent grade and freshly distilled water were used for all HPLC analyses. Other solvents were of analytical-reagent grade. The reference compounds used were isolated from licorice root and characterized in our laboratory by spectroscopic methods. Each compound shows a single peak in HPLC. Their melting points are as follows: liquiritin, 213–215°C (lit.[14], 212°C); isoliquiritin, 188–190°C (lit.[15], 187–189°C); liquiritigenin, 203–205°C (lit.[16], 201–203°C); isoliquiritigenin, 198–200°C (lit.[16], 198–200°C); glycycoumarin, 236–238°C (lit.[16], 231–233°C); isoglycycoumarin, 236–237°C (lit.[17], 236–237°C); licochalcone A, 102–103°C (lit.[18], 102°C); glycyrol, 247–249°C (lit.[19], 243–245°C); and isoglycyrol, 301–303°C (lit.[19], 298–300°C).

Chromatographic conditions

The temperature of the column oven was 28–30°C. The programme for the mobile phase, flow-rate and UV detector wavelength is given in Table I.

Analytical procedure

A 100-mg amount of powdered crude drug was weighed into a micro-Soxhlet extractor and refluxed with 10 ml of methanol for 2 h in a water-bath (70°C). The methanolic extract was concentrated at 70°C to less than 3 ml, transferred to a measuring flask and, after cooling to room temperature, was made up to 5 ml with methanol. Volumes of 4–10 μl of this solution were used for HPLC analysis.

RESULTS AND DISCUSSION

Selection of separation system

In order to separate the five flavonoids and four coumarins, we tried a number of columns, *viz.*, MicroPak MCH-5 (15 cm × 4.6 mm I.D.) (Varian), Zorbax ODS (15 cm x 4.6 mm I.D.) (Shimadzu) and μBondapak CN (30 cm × 7.8 mm I.D.)

TABLE I

PROGRAMME FOR MOBILE PHASE COMPOSITION, FLOW-RATE AND UV DETECTOR WAVELENGTH FOR QUANTITATIVE ANALYSIS OF LICORICE ROOT BY HPLC

Time (min)	*Mobile phase (%, v/v)*		*Flow-rate (ml/min)*	*UV detector (nm)*
	3% acetic acid	*Acetonitrile*		
0 (start)	80	20	1.0	310
13.0	↓	↓	↓	365
15.0	50	50	1.2	365
25.0	20	80	1.2	365
30 (stop)	20	80	1.2	365

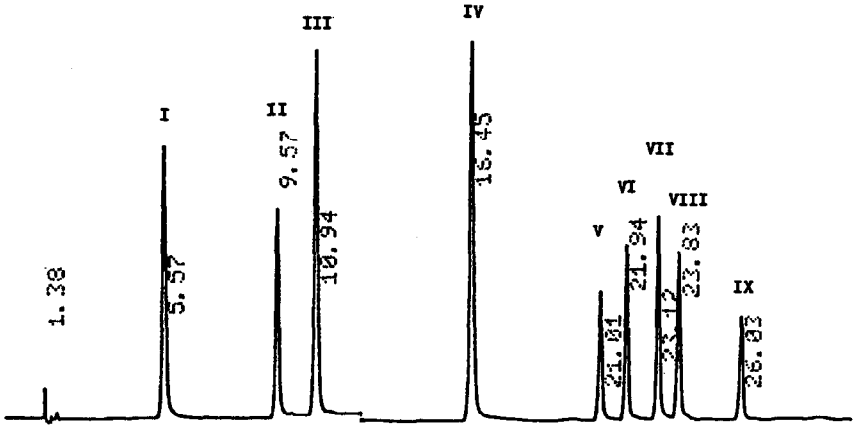


Fig. 2. HPLC of compounds I–IX. Numbers at peaks indicate retention times in min.

(Waters Assoc.), and several gradient elution systems, *i.e.*, methanol–water, methanol–water–acetic acid and acetonitrile–3% acetic acid and found that the Zorbax ODS column combined with acetonitrile–3% acetic acid gives the best separation and resolution for all nine compounds in 30 min (Table I and Fig. 2).

Selection of wavelength

The maximum absorption wavelengths (nm) and intensities (log ε) of the nine compounds are as follows: liquiritin, 220 (4.10), 275 (4.00), 312 (3.75); isoliquiritin, 232 (4.20 sh), 362 (4.43); liquiritigenin, 231 (4.33), 275 (4.24), 312 (3.95); isoliquiritigenin, 262 (4.10 sh), 365 (4.45); glycycoumarin, 252 (4.36), 258 (4.30), 352 (4.60); isoglycycoumarin, 253 (4.28), 256 (4.24), 353 (4.48); licochalcone A, 264 (3.79), 304 (3.94), 370 (4.20); glycyrol 231 (4.49), 243 (4.36), 347 (4.42), 353 (4.38 infl.); and isoglycyrol, 224 (4.38 sh), 247 (4.34), 347 (4.40), 363 (4.36 infl.). Further, other substances in licorice can interfere with the detection, such as the coexisting glycyrrhizin-

TABLE II

REGRESSION EQUATIONS AND CORRELATION COEFFICIENTS FOR COMPOUNDS I TO IX

Function: $y = ax + b$, where y = peak area and x = amount (μg).

Compound	*Regression equation*	*Correlation coefficient*
I	$y = 83.49x + 2.23$	0.9935
II	$y = 155.33x - 1.12$	0.9989
III	$y = 87.29x - 0.23$	0.9979
IV	$y = 685.24x - 0.23$	0.9983
V	$y = 246.32x + 0.47$	0.9969
VI	$y = 269.62x - 0.61$	0.9985
VII	$y = 1586.12x - 0.91$	0.9978
VIII	$y = 313.31x - 1.08$	0.9988
IX	$y = 352.91x - 0.47$	0.9985

ic acid, which shows a retention time close to that of isoliquiritigenin in our analytical system. Considering the above factors, we chose wavelengths of 310 and 365 nm for detection, not only to avoid the interference from glycyrrhizinic acid, which gives a maximum absorption at 248 nm ($\log \varepsilon = 2.16$), but also to make the baseline of the chromatogram smooth. The programme for the mobile phase, flow-rate and UV detector wavelength for the analysis of the above nine compounds is given in Table I.

Linearity and precision

In order to check the linearity of the relationship between amount of compound and peak area using the above separation system, suitable amounts of each of the nine compounds were weighed and mixed in a measuring flask, dissolved and suitably diluted with methanol to serve as a standard solution. Various amounts of the standard solution were injected and chromatographed. The regression equation $y = ax + b$, where x is the amount of compound (μg) and y is the peak area (absorbance $\times 10^{-3}$), and the correlation coefficients of compounds **I–IX** are given in Table II. All the graphs exhibit good linearity and obey Beer's law. The concentration ranges for a linear relationship between amount and peak area are as follows (all 10^{-4} μmol): liquiritin, 2.536–12.68; isoliquiritin, 2.244–11.22; liquiritigenin, 3.203–16.02; isoliquiritigenin, 0.9766–4.883; glycycoumarin, 0.6576–6.576; isoglycycoumarin, 0.8696–13.9; licochalcone A, 0.1746–2.793; glycyrol, 0.7104–11.37; and isoglycyrol, 0.4180–6.667. Volumes of 10 μl of the mixed solution of the nine compounds were chromatographed and the experiments repeated six times. The results showed that the relative standard deviations for the nine compounds were between 1.38 and 3.70%.

Selection of extraction methods

Four methods of extracting the flavonoids and coumarins from licorice root were compared for efficiency. Amounts of 100 mg of the powdered drug were weighed and extracted by one of the following methods: (i) hot reflux with ethyl acetate in a

TABLE III

EFFICIENCY OF DIFFERENT METHODS FOR EXTRACTING COMPOUNDS I–IX FROM *GLYCYRRHIZA URALENSIS* ROOT

Compound	*Amount of compound extracted (%)*			
	Hot ethyl acetate reflux	*Hot ethanol reflux*	*Cold methanol maceration*	*Hot methanol reflux*
I	0.481	0.918	1.016	1.152
II	0.444	0.770	0.843	0.949
III	0.384	0.697	0.666	0.662
IV	0.082	0.120	0.114	0.136
V	0.048	0.048	0.041	0.048
VI	Trace	Trace	Trace	0.002
VII	Trace	Trace	Trace	Trace
VIII	0.016	0.018	0.014	0.019
IX	0.003	0.003	Trace	0.003
Total	0.458	2.574	2.694	2.971

TABLE IV

RECOVERIES OF COMPOUNDS I–IX FROM LICORICE ROOT (*GLYCYRRHIZA URALENSIS* FISCH.)

Compound	*Added (mg)*	*Recovered (mg)*	*Recovery (%)*	*Mean recovery ± S.D. (%)*	*Relative standard deviation (%)*
I	1.060	1.055	99.5	96.2 ± 2.96	3.08
	1.590	1.489	93.7		
	2.385	2.278	95.5		
II	0.992	0.998	100.6	99.1 ± 2.86	2.89
	1.488	1.426	95.8		
	2.323	2.252	100.9		
III	0.820	0.827	100.9	99.1 ± 3.06	3.08
	1.230	1.176	95.6		
	1.845	1.862	100.9		
IV	0.250	0.252	100.7	100.3 ± 0.72	0.72
	0.375	0.373	99.5		
	0.563	0.568	100.8		
V	0.386	0.362	93.8	95.6 ± 1.70	1.70
	0.579	0.554	95.7		
	0.869	0845	97.2		
VI	0.512	1.515	100.6	101.7 ± 1.92	1.79
	0.768	0.797	103.8		
	1.152	1.160	100.7		
VII	0.094	0.098	104.1	101.5 ± 4.30	4.24
	0.141	0.136	96.5		
	0.212	0.220	103.8		
VIII	0.416	0.414	99.5	99.4 ± 0.153	0.62
	0.624	0.620	99.4		
	0.936	0.929	99.2		
IX	0.244	0.255	104.5	105.2 ± 0.651	0.62
	0.366	0.385	105.2		
	0.549	0.581	105.8		

TABLE V

CONTENTS OF COMPOUNDS I–IX IN THREE SPECIES OF CHINESE LICORICE ROOT (%)

Compound	*G. uralensis*[a]	*G. uralensis*[b]	*G. inflata*	*G. glabra*
I	3.649	1.152	0.593	0.470
II	2.328	0.949	0.508	0.425
III	0.121	0.662	0.032	0.014
IV	0.121	0.136	0.016	0.016
V	0.138	0.048	0.017	0.011
VI	0.018	0.002	0.009	0.005
VII	Trace	Trace	0.138	0.025
VIII	0.044	0.019	Trace	0.008
IX	0.027	0.003	Trace	Trace
Total	6.446	2.971	1.313	0.974

[a] From Shanxi.
[b] From Nei Monggol.

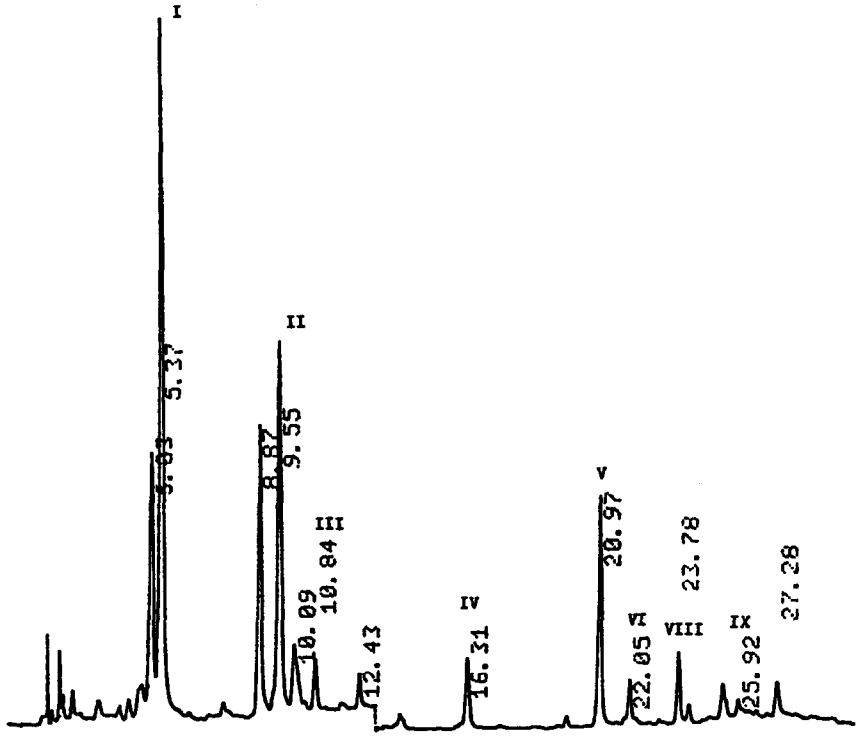


Fig. 3. HPLC of Chinese licorice root (*Glycyrrhiza uralensis* Fisch.). Numbers at peaks indicate retention times in min.

micro-Soxhlet extractor in a water-bath (80°C) for 2 h; (ii) hot reflux with ethanol in a micro-Soxhlet extractor in a water-bath (85°C) for 2 h; (iii) cold maceration with methanol in a stoppered centrifuge tube at room temperature for 24 h and (iv) hot reflux with methanol in a micro-Soxhlet extractor in a water-bath (70°C) for 0.5, 1.0, 1.5 and 2 h. The results showed that method iv (hot reflux with methanol for 2 h) is the best (Table III).

Recovery of compounds ***I–IX***

Suitable amounts of compounds **I–IX** were added to a sample of powdered licorice root with known contents of the nine compounds and the whole was extracted and analysed by the above procedure. The recoveries of the flavonoids and coumarins were 95.6–105.2% with relative standard deviations of 0.62–4.24% (Table IV).

Contents of compounds ***I–IX*** *in licorice root*

In order to demonstrate the validity of this method, the contents of the nine compounds in three species of Chinese licorice root were determined. The peaks were checked by adding a mixed solution of reference compounds to the sample solution before injection. The results are given in Table V. A chromatogram of the extract of *G. uralensis* root is illustrated in Fig. 3.

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