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Application of Ion-Pair High-Performance Liquid Chromatography to the Analysis of Glycyrrhizin in Glycyrrhizae Radix

KAZUHIKO SAGARA,* YUJI ITO, TOSHIYUKI OSHIMA, MASANARI KAWAURA and TETSUO MISAKI

Research Center, Taisho Pharmaceutical Co., Ltd., 1-403, Yoshino-cho, Omiya-shi, Saitama 330, Japan

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A new, simple and precise method using ion-pair high-performance liquid chromatography was developed for the determination of glycyrrhizin in Glycyrrhizae Radix. A reversed-phase system was used, consisting of an ODS column with water and methanol (45 : 55) containing 5 mM tetra-*n*-butylammonium hydroxide and adjusted to pH 6.0 by phosphoric acid as the mobile phase. A peak (peak I) which had the same retention time as glycyrrhizin in the established ion-suppression method was separated by the ion-pair method.

The analytical results of glycyrrhizin measured by this method were lower by 6.2% to 10.8% than those obtained by the ion-suppression method.

Keywords——ion-pair high-performance liquid chromatography; Glycyrrhizae Radix; glycyrrhizin; crude drug

Glycyrrhizae Radix, known as licorice root in Europe, has been used extensively in oriental medicine (referred to as Kanpo). Glycyrrhizin, which is the main component of Glycyrrhizae Radix, is well known as a sweet-tasting compound and its pharmacological activity has been widely investigated. The analysis of glycyrrhizin is important in quality estimation of Glycyrrhizae Radix.

Several high-performance liquid chromatographic (HPLC) methods using anion-exchange[1-4] and reversed-phase[5-8] columns for the analysis of glycyrrhizin in Glycyrrhizae Radix have been reported; the reversed-phase chromatographic methods using chemically modified silica gel, for instance, octadecylsilane (ODS)-silica gel, are superior to the anion-exchange chromatographic methods, especially as regards theoretical plates and peak separation. There have been several reports published on gradient methods[5,8] and ion-suppression methods[6-8] using a reversed-phase column. The gradient methods were developed for the simultaneous determination of glycyrrhizin and glycyrrhetic acid, and for the separation of various components in Glycyrrhizae Radix. These methods, however, require too much time for repeated analysis because the solvent constitution of the mobile phase has to be restored to the initial condition. Although the ion-suppression method of Okada *et al.*[7] can be used for the analysis of only glycyrrhizin, it seems to be simpler and more rapid than the gradient method for the analysis of many samples.

In recent years, the reversed-phase ion-pair chromatographic method has been increasingly used for the analysis of ionic compounds since the capacity factor of the ionic compounds is increased by forming ion complexes with the pairing reagent. This technique has also been applied to the analysis of some natural products in plants, for example, berberine alkaloids,[9] aconitine alkaloids[10] and ephedrine alkaloids[11] as basic compounds, and sennosides[12] and flavone glucuronides[13] as acidic compounds.

In this paper, we report the development of a simple and precise method for determination of glycyrrhizin in Glycyrrhizae Radix by utilizing the ion-pair technique. The

results are also compared with those of the ion-suppression method.[7]

Experimental

Plant Material——Commercial Glycyrrhizae Radix was purchased from Alps Pharmaceutical Ind. Co., Ltd. (Gifu-ken).

Reagents——Glycyrrhizin monoammonium was purified by recrystallization from 85% ethanol after fractionation of commercial glycyrrhizin monoammonium by preparative HPLC according to Okada's method.[7] Tetra-*n*-butylammonium hydroxide was purchased from Tokyo Kasei Co., Ltd. Methanol used for chromatography was of special grade.

Chromatographic Conditions——A Hitachi model 635 liquid chromatograph equipped with a Ubilog-5 IV UV-spectrophotometer was used. Peak area was calculated by a SIC intelligent integrator, model 7000 A. A stainless-steel column (150 mm × 4 mm i.d.) packed with ODS chemically bonded silica gel (TSK gel LS-410, 5 μm, Toyo Soda Co., Ltd.) was used. A mixture of water and methanol (45 : 55) containing 5 mM tetra-*n*-butylammonium hydroxide and adjusted to pH 6.0 by phosphoric acid was used as the mobile phase. The analysis was carried out at 50 °C at a flow rate of 1.0 ml/min. The substances eluted were detected by a UV detector at a wavelength of 254 nm.

Assay Procedure——About 0.5 g of Glycyrrhizae Radix dry powder was weighed accurately, placed in 30 ml of the mobile phase for HPLC and refluxed on a water bath at 85 °C. After cooling, it was centrifuged and decanted. The residue was washed twice with 10 ml of the mobile phase. The extract and washings were placed in a 50 ml volumetric flask and diluted to 50 ml with the mobile phase. Ten microliters of this solution was injected into the HPLC column. The glycyrrhizin content in Glycyrrhizae Radix was calculated from the peak area.

Calibration Curve and Detection Limit——The calibration curve for glycyrrhizin was obtained from 52.45 to 734.3 μg/ml. The regression equation was as follows: $y = 14276X - 164$ ($r = 0.999$), where y is the peak area and X is the concentration (μg/ml). The detection limit was 20 ng at a signal-to-noise ratio of 3 : 1 for the peak height.

Results and Discussion

Effects of Methanol, Counter-Ion and pH on the Capacity Factor of Compounds

The mixing ratio of methanol to water, the kind and concentration of counter-ion and the pH of the mobile phase were varied to find the optimum conditions for separation of glycyrrhizin in Glycyrrhizae Radix.

Tetraethyl-(TEA), tetra-*n*-propyl-(TPA), tetra-*n*-butyl-(TBA) and tetra-*n*-amylammonium (TAA) salts were examined as the counter-ion. After a counter-ion had been added to the water–methanol (45 : 55) mobile phase at a final concentration of 5 mM, the mixtures were adjusted to pH 6.0. As shown in Fig. 1, the capacity factor (k') of glycyrrhizin increased in proportion to the length of the alkyl chain of the counter-ions. Glycyrrhizin was eluted at an appropriate time for analysis and with a good separation from other components when TBA was used, whereas in the case of TAA it was not eluted within 2 h. TBA concentration in the mobile phase was varied from 0 to 20 mM (Fig. 2). The amount of TBA increased the capacity factor of glycyrrhizin concentration-dependently, and 5 mM TBA was selected for this analysis.

The pairing effect occurs when a molecule dissociates into ions in the mobile phase. Among the three carboxylic groups in glycyrrhizin, two glucuronic acids dissociate near pK_a 3.6 and the carboxylic acid of the aglycone dissociates near pK_a 4.9.[14] Therefore, pH 6.0 was selected for the mobile phase containing 5 mM TBA since all acid moieties of glycyrrhizin seemed to dissociate.

Determination of Glycyrrhizin in Glycyrrhizae Radix

The mobile phase was selected as the extraction solvent because the ion-pair mobile phase shows good efficiency for the extraction of components from crude drugs;[9,11,13] in this case the mobile phase showed extraction efficiency equal to those of other solvents which were previously reported.[1-8] The reproducibility of this method is shown in Table I.

Fifteen lots of Glycyrrhizae Radix from different countries were analyzed by the ion-pair method, and three chromatograms are shown in Fig. 3. The ion-suppression method[7] [the

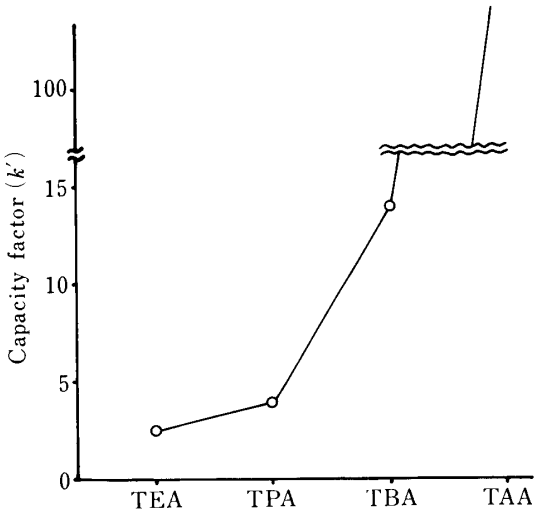

Fig. 1. Effect of Various Counter-Ions on the Capacity Factor (k')

Solute, ○, glycyrrhizin. Counter-ion: TEA, tetraethylammonium salt; TPA, tetra-*n*-propylammonium salt; TBA, tetra-*n*-butylammonium salt; TAA, tetra-*n*-amylammonium salt. Flow rate, 1 ml/min. Temperature, 50 °C. The mobile phase was a mixture of water and methanol (45 : 55) adjusted to pH 6.0 by adding phosphoric acid.

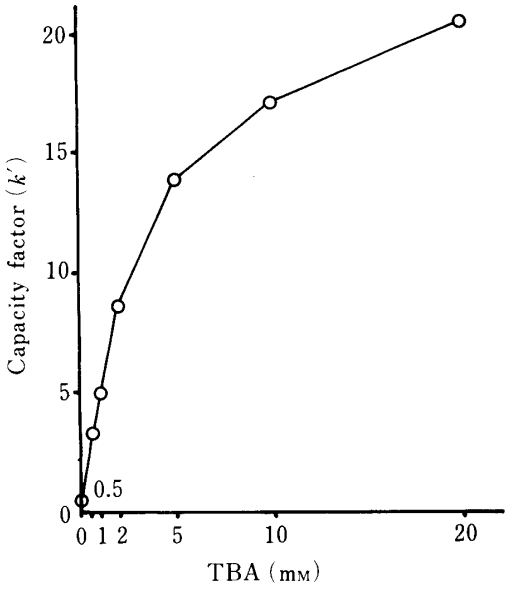

Fig. 2. Effect of TBA Concentration on the Capacity Factor (k')

Solute, ○, glycyrrhizin. HPLC conditions, see Fig. 1.

TABLE I. Reproducibility of Analytical Values

Sample	Glycyrrhizin (%)
China (Seihoku)	4.79
China (Seihoku)	4.79
China (Seihoku)	4.68
China (Seihoku)	4.83
China (Seihoku)	4.79
$\bar{X}_5$	4.78
C.V.	1.18

C.V., coefficient variation.

mobile phase consisted of 2% acetic acid and acetonitrile (2 : 1)] was also applied to them to ascertain the reliability of the analytical results obtained by the ion-pair method. As shown in Table II, the contents of glycyrrhizin measured by the ion-pair method were from 1.05% in the sample from Spain to 7.58% in that from Tohoku (China). However, they were lower by 6.2 to 10.8% than those obtained by the ion-suppression method. This result suggests that the glycyrrhizin peak eluted under the ion-suppression conditions contains some other components as an impurity. To confirm this, a part of the glycyrrhizin collected by the ion-suppression method was injected onto the column under the ion-pair conditions. As shown in Fig. 4, the single peak of glycyrrhizin obtained by the ion-suppression method was clearly separated into two peaks. The amounts of the impurity peak (peak I) just corresponded to the differences between the analytical values obtained by the ion-pair method and the ion-suppression method.

On the other hand, when the part of the glycyrrhizin collected by the ion-pair method was further analyzed under the ion-suppression conditions, there was only one peak on the chromatogram. Moreover, although glycyrrhizin was eluted at about 10 min under the ion-

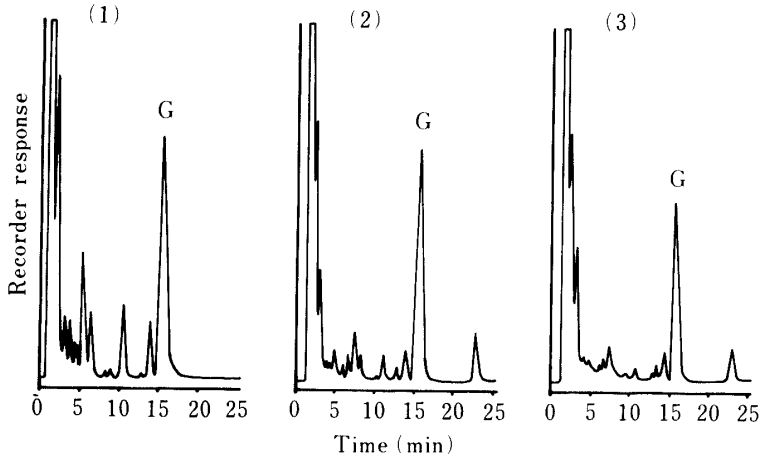

Fig. 3. Chromatograms of Glycyrrhizin in Glycyrrhizae Radix by the Ion-Pair Method

Chromatograms, (1), China (Seihoku); (2), Russia; (3), Afghanistan. Peak, G= glycyrrhizin.

TABLE II. Comparison of the Ion-Suppression Method and Ion-Pair Method for Determination of Glycyrrhizin in Glycyrrhizae Radix

Sample	Ion-pair (%)	Ion-suppression (%)	Ratio[a] (%)
China (Seihoku)	4.78	5.33	89.7
China (Seihoku)	2.49	2.79	89.2
China (Seihoku)	5.90	6.44	91.6
China (Tohoku)	7.58	8.37	90.6
China (Tohoku)	5.16	5.67	91.0
China (Tohoku)	6.27	6.90	90.9
China (Shinkyo)	4.75	5.15	92.2
China (Shinkyo)	2.11	2.34	90.2
Russia	4.88	5.26	92.8
Afghanistan	3.41	3.72	91.7
Italy	1.20	1.33	90.2
Italy	2.23	2.38	93.7
Spain	1.05	1.12	93.8
Iran	4.51	4.83	93.4
Pakistan	3.35	3.74	89.6

a) Analytical value by the ion-pair method/analytical value by the ion-suppression method.

suppression conditions, even when the elution time of glyçyrrhizin was delayed by decreasing the organic modifier content in the mobile phase, glycyrrhizin and peak I were not separated.

To determine the effect of the hydrophobic counter-ion and to examine the difference between the ion-pair effect and ion-suppression effect, two kinds of mobile phases, which contained TBA and ammonium phosphate as the smallest quaternary ammonium counter-ion, were selected. The pH values were changed between 3 and 8. The relationships between pH and the capacity factor are shown in Fig. 5. The behaviors of glycyrrhizin and peak I were almost the same regardless of the kind of counter ion below pH 3.5 where the ion-suppression effect would be induced, but were different above pH 3.5. In the mobile phase containing TBA, ion-pair effect occurred from pH 3.5 and the capacity factors of glycyrrhizin and peak I increased gradually. They were not separated below pH 4.3, but were separated from pH 4.6. The maximum was at pH 5.0, after which the capcity factors decreased. The decrease of the capacity factors seems to be due to the increase of phosphoric acid concentration, and the

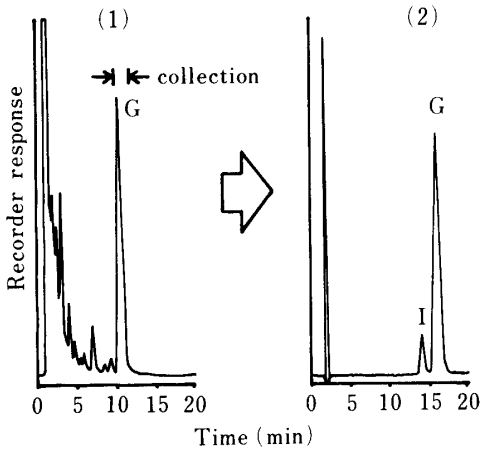

Fig. 4. Analysis of Glycyrrhizin Fraction Obtained by the Ion-Suppression Method

Mobile phase (1), a mixture of 2% acetic acid and acetonitrile (2:1); (2), a mixture of water and methanol (45:55) containing 5 mM TBA and adjusted to pH 6.0 by adding phosphoric acid.

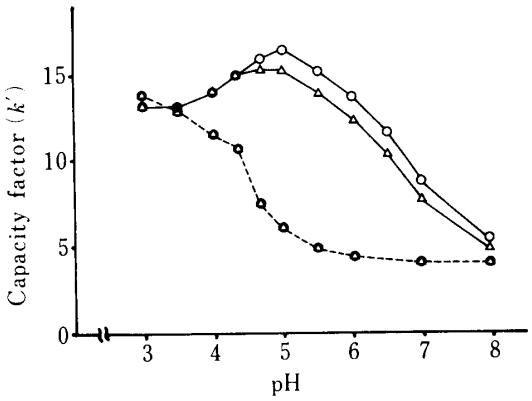

Fig. 5. Relationship between pH and Capacity Factor with Two Mobile Phases Containing Different Counter-Ions

Solutes: ○, glycyrrhizin; △, peak I. Counter-ions: ——, 5 mM TBA; – – –, 5 mM ammonium phosphate. Other conditions, see Fig. 1.

consequent masking effect on silanol groups on ODS. On the other hand, in the case of the mobile phase containing ammonium phosphate, the capacity factors of glycyrrhizin and peak I decreased between pH 3.5 and 5.5, and remained almost constant above pH 5.5 with no separation. It appears that formation of the ion-pair complex with the hydrophobic counter-ion is required for the separation of glycyrrhizin and peak I.

As described above, peak I, which had not been observed by the ion-suppression method, was found, and the analytical results for glycyrrhizin obtained under the ion-suppression conditions were confirmed to be somewhat higher than those obtained by the ion-pair method; the latter method seems to be more accurate for the determination of glycyrrhizin in Glycyrrhizae Radix. This method should also be useful as a routine method for the quality estimation of Glycyrrhizae Radix since the analytical time is short.

The application of this ion-pair method to complicated preparations and further studies on the structure of peak I are in progress, and the results will be reported in the near future.

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