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SIMULTANEOUS DETERMINATION OF BAICALEIN, WOGONIN, OROXYLIN-A AND THEIR GLUCURONIDES IN SCUTELLARIAE RADIX BY ION-PAIR HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

A new, simple and precise analytical method using ion-pair high-performance liquid chromatography was developed for simultaneous determination of six flavonoids, namely baicalin, baicalein, wogonin, wogonin-glucuronide, oroxylin-A and oroxylin-A-glucuronide, in Scutellariae Radix. A reversed-phase chromatographic system consisting of a chemically bonded ODS silica gel column and water–acetonitrile (68:32), containing 5 m*M* tetra-*n*-amylammonium bromide and adjusted to pH 4.0 by phosphoric acid, as mobile phase was used. The six flavonoids in this crude drug were completely separated within 25 min. Oroxylin-A and its glucuronide were separated for the first time. The analytical results for various samples are described.

INTRODUCTION

The crude drug, Scutellariae Radix, is well known and is frequently used in oriental pharmaceutical preparations, kanpo. It contains large amounts of flavonoids such as baicalein, wogonin, oroxylin-A and their glycosides[1–8], and the main glycosides are glucuronides. Attention has been paid to the flavonoids in Scutellariae Radix ever since their anti-allergic activity was found[9]. The activities of baicalin, which is the main component, baicalein and wogonin, in particular, have been investigated. From the results it has been concluded that the most potent anti-allergic material is baicalein while other flavonoids have low activities. Therefore the quantitative analysis of individual flavonoids is important to evaluate the quality of Scutellariae Radix.

Although some UV[10], thin-layer chromatography (TLC)[10,11] and gas–liquid chromatography (GLC)[12] methods for the analysis of flavonoids in this crude drug have been reported, they are mainly for baicalin and baicalein and involve time-consuming procedures such as the combination of preparative TLC and UV and

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silylation for GLC. High-performance liquid chromatography (HPLC) methods have also been examined using gradient[13] and ion-suppression techniques[14] to separate baicalin, baicalein, wogonin-glucuronide and wogonin. However, these two methods also require long times to determine aglycones and their glucuronides simultaneously due to their different polarities. Moreover, under conditions appropriate for the analysis of the aglycones, the glucuronides are eluted very early and with poor separation from each other and other components of Scutellariae Radix. Therefore, in order to determine the aglycones and their glucuronides simultaneously, it is necessary that both sets of compounds are retained on the column.

Recently, ion-pair chromatography has been applied to the analysis of some natural products in plants, for example, berberine[15], aconitine [16] and ephedrine alkaloids[17] as basic compounds and sennosides[18] and glycyrrhizin[19] as acidic compounds, because it results in appropriate capacity factors for complexes formed with the counter ion. Accordingly, ion-pair HPLC was examined for the simultaneous determination of baicalein, baicalin, wogonin, wogonin-glucuronide, oroxylin-A and oroxylin-A-glucuronide in Scutellariae Radix.

EXPERIMENTAL

Plant materials

Commercial Scutellariae Radix samples were purchased from Matsuura Yakugyo.

Apparatus

A Hitachi Model 655 liquid chromatograph equipped with an Ubilog-51V UV-spectrophotometer and a stainless-steel column (150 × 4 mm I.D.) packed with chemically bonded ODS silica gel (TSK gel LS-410, 5 μm, Toyo Soda) was used.

Reagents

Baicalein, baicalin, wogonin, wogonin-glucuronide, oroxylin-A and oroxylin-A-glucuronide were isolated from Scutellariae Radix as described[1–8], purified by HPLC and recrystallized. Tetramethyl-, tetraethyl-, tetra-*n*-propyl, tetra-*n*-butyl- and tetra-*n*-amylammonium bromide were purchased from Tokyo Kasei, and tetra-*n*-heptylammonium bromide from Aldrich. The acetonitrile used for the chromatography was a special grade.

HPLC conditions

Water–acetonitrile (68:32), containing 5 m*M* tetra-*n*-amylammonium bromide and adjusted to pH 4.0 by phosphoric acid, was used as the mobile phase. The column temperature was maintained at 30°C and the flow-rate was 1.0 ml/min. The substances eluted were detected by a UV detector operated at a wavelength of 280 nm.

Assay procedure

Scutellariae Radix dry powder (0.5 g) places in 30 ml of the mobile phase for HPLC, then heated under reflux on a water-bath at 85°C. After cooling, it was centrifuged at 3000 rpm and decanted. The residue was washed twice with 10-ml portions of the mobile phase. The extract and washings were placed in a 50-ml volumetric

flask and diluted to 50 ml in the mobile phase. A 10-μl volume of this solution was injected for HPLC. The content of each flavonoid in Scutellariae Radix was calculated from the relevant peak height.

Calibration curves and detection limits

The calibration curves for baicalein, baicalin, wogonin, wogonin-glucuronide, oroxylin-A and oroxylin-A-glucuronide were obtained over the conentration ranges 5.75–50.36, 13.10–262.0, 1.27–16.93, 14.66–58.65, 5.00–20.00 μg/ml and 5.13–25.63 μg/ml. The corresponding regression equations were as follows: $y = 0.150x - 0.645$ ($r = 0.999$); $y = 0.158x + 0.139$ ($r = 0.999$); $y = 0.172x + 0.024$ ($r = 0.999$); $y = 0.150x + 0.036$ ($r = 0.999$); $y = 0.132x - 0.007$ ($r = 0.999$) and $y = 0.143x + 0.035$ ($r = 0.999$), respectively. The detection limits were 6, 25, 10, 25, 8 and 35 ng respectively, at a signal-to-noise ratio of 3:1 for the peak heights.

RESULTS AND DISCUSSION

HPLC conditions

Elution parameters such as organic content of the mobile phase, kind and concentration of the counter ion, pH and column temperature were varied to find the optimum elution conditions on chemically bonded ODS silica gel.

Tetraethyl-, tetra-*n*-propyl-, tetra-*n*-butyl-, tetra-*n*-amyl- and tetra-*n*-heptylammonium bromide were examined as counter ions for the separation of each flavonoid, under the condition that the aglycones were eluted between 9 and 25 min. After each counter ion had been added to the water–acetonitrile (68:32) mobile phase in a final

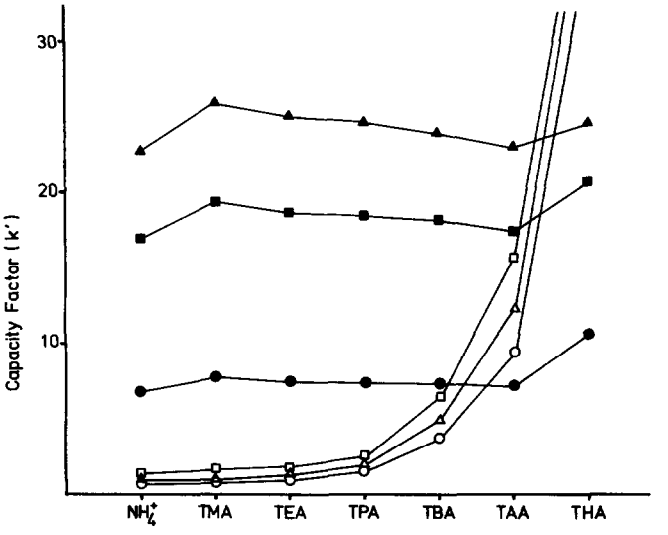

Fig. 1. Effect of counter ions on the capacity factors, k', of baicalin (○), baicalein (●), wogonin glucuronide (□), wogonin (■), oroxylin-A-glucuronide (△) and oroxylin-A (▲). Counter ions: NH_4^+ = ammonium; TMA = tetramethylammonium bromide; TEA = tetraethylammonium bromide; TPA = tetra-*n*-propylammonium bromide; TBA = tetra-*n*-butylammonium bromide; TAA = tetra-*n*-amylammonium bromide; THA = tetra-*n*-heptylammonium bromide. Flow-rate: 1 ml/min. Temperature: 30°C. Mobile phase: water–acetonitrile (68:32) adjusted to pH 4.0 by phosphoric acid.

concentration of 5 m*M*, the mixtures were adjusted to pH 4.0 by phosphoric acid. As shown in Fig. 1, the capacity factors of the glucuronides increased in proportion to the length of the alkyl chain of the counter ions. Each glucuronide was eluted between baicalein and wogonin with a good peak separation when tetra-*n*-amylammonium bromide (TAA) was used, while the capacity factors of the aglycones were little altered. This result suggests that the aglycones do not form ionic complexes with the counter ion under these conditions. Thus, TAA was suitable as the counter

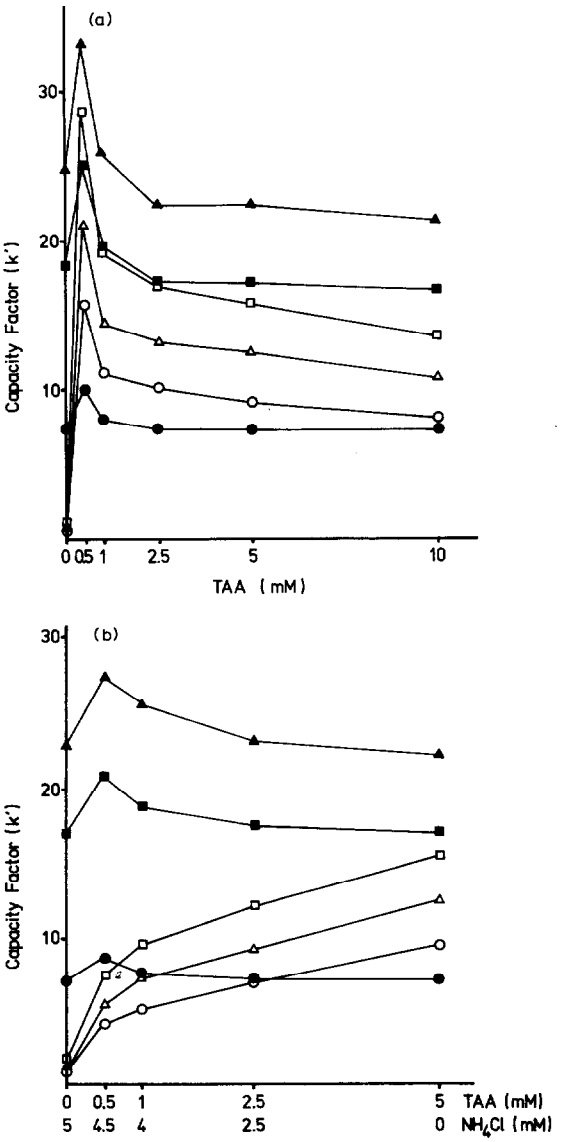

Fig. 2. Effect of TAA concentration on the capacity factor, *k'*. For solutes and chromatographic conditions see Fig. 1.

ion for the simultaneous determination of the aglycones and their glucuronides in Scutellariae Radix by ion-pair HPLC.

The TAA concentration in the mobile phase was varied from 0 to 10 m*M*. However, a decrease in the TAA concentration unexpectedly caused an increase in the capacity factors of both the glucuronides and the aglycones, so with the latter (Fig. 2a). This phenomenon seemed to be due to the adsorption of these substances to remaining silanol groups on ODS upon decreasing the total salt concentration. To elucidate the effect of TAA, ammonium chloride was added to each mixture containing TAA at different concentrations to give a final salt concentration of 5 m*M*. The capacity factors of the glucuronides again showed a dependence on the concentration of TAA (Fig. 2b).

Fig. 3 shows the effect of the acetonitrile concentration in the mobile phase on the capacity. The acetonitrile concentration not only affected the capacity factors of the flavonoids, but also influenced the degree of variation between the glucuronides and the aglycones. A concentration of 32% acetonitrile was selected for the best separation under the condition that the glucuronides were eluted between baicalein and wogonin.

The capacity factors of the glucuronides are also strongly influenced by the mobile phase pH because the ion-pair effect involves the formation of ionic complexes with the counter ion. The relationship between the capacity factors of the flavonoids and the mobile phase pH is shown in Fig. 4. Although all the glucuronides were rapidly eluted as a group at pH 2.3 where the formation of ionic complexes was incomplete due to suppression of the dissociation of glucuronic acid, they were retained on the column by increasing the mobile phase pH. A pH of 4.0 was best for the separation of each flavonoid, and resulted in reproducible retention times. Con-

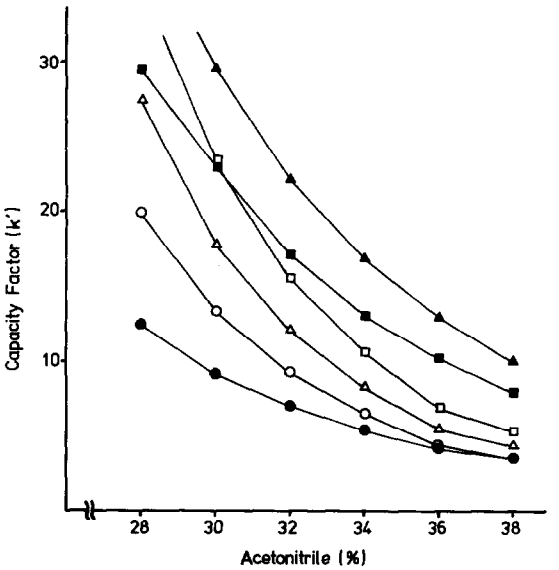

Fig. 3. Effect of acetonitrile concentration on the capacity factor, *k'*. Mobile phase: water–acetonitrile containing 5 m*M* TAA and adjusted to pH 4.0 by phosphoric acid. Solutes and other conditions as in Fig. 1.

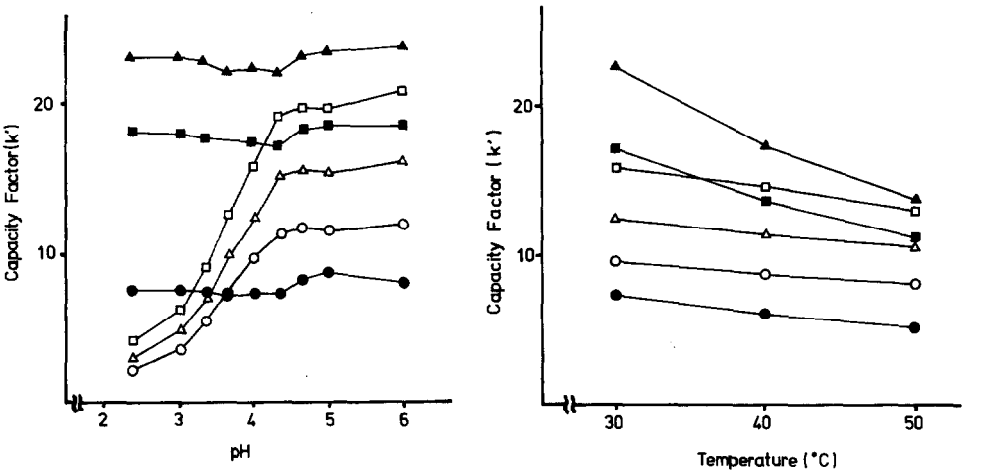

Fig. 4. Effect of pH on the capacity factor, k'. Mobile phase: water–acetonitrile (68:32) containing 5 m*M* TAA. Solutes and other conditions as in Fig. 1.

Fig. 5. Effect of column temperature on the capacity factor, k'. Solutes and chromatographic conditions as in Fig. 1.

trary to the behaviour of the glucuronides, the aglycones were hardly influenced by pH. This result is reasonable in that the aglycones do not form ionic complexes with the counter ion over this pH range.

In contrast to the effect of the acetonitrile concentration, the effect of the column temperature on the degree of variation of the aglycones was much greater than that on the glucuronides (Fig. 5).

As a result of these experiments, water–acetonitrile (68:32), containing 5 m*M* TAA and adjusted to pH 4.0 by phosphoric acid, was selected as the mobile phase. The chromatogram of the six standards is shown in Fig. 6.

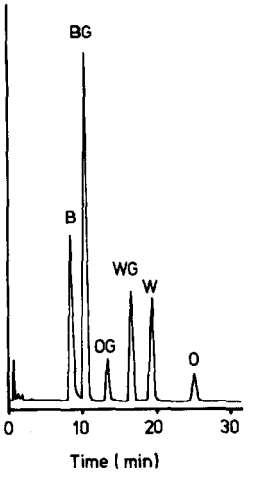

Fig. 6. Chromatogram of standards. Peaks: B = baicalein; BG = baicalin; OG = oroxylin-A-glucuronide; WG = wogonin-glucuronide; W = wogonin; O = oroxylin-A.

TABLE I

EFFECT OF SOLVENTS ON EXTRACTION EFFICIENCY (%)

B = Baicalein; BG = baicalin; W = wogonin; WG = wogonin-glucuronide; O = oroxylin-A; OG = oroxylin-A-glucuronide.

Solvent	*B*	*BG*	*W*	*WG*	*O*	*OG*
Mobile phase	100.0	100.0	100.0	100.0	100.0	100.0
Methanol	93.5	77.9	104.4	91.0	100.8	91.6
Water	77.7	74.3	80.2	81.0	70.6	76.5
Phosphate buffer, pH 6.0	37.9	70.2	78.6	79.5	73.4	73.1
32% Acetonitrile (pH 4.0)	96.1	100.0	98.8	99.0	100.0	98.4

Extraction conditions

On the basis of our previous experiments[15,17] especially regarding the glucuronides, it was expected that higher extraction efficiences would be obtained by use of a mobile phase containing TAA. Table I shows the extraction efficiency of various solvents, namely water, phosphate buffer (pH 6.0), methanol, the present mobile phase and the mobile phase without TAA. The last solvent was chosen so as to determine the effect of TAA on the extraction whereas the other solvents, which had been used in earlier methods[12–14], were included for comparison with the mobile phase. The extraction efficiencies of water and the phosphate buffer were about 20–30% lower than that of the mobile phase for all the flavonoids, and even with methanol it was about 10–20% lower for the glucuronides. On the other hand, the mobile phase without TAA extracted all the flavonoids as efficiently as the mobile phase. From these results it seemed that the extraction of the flavonoids is based on the mixture of water and acetonitrile rather than TAA.

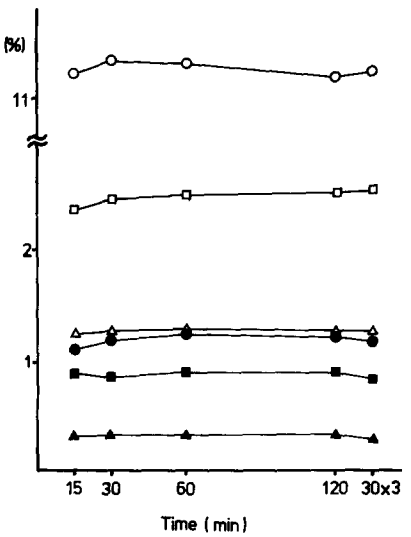

Fig. 7. Efficiencies of extraction of the flavonoids from Scutellariae Radix with the mobile phase at various times. Solutes as in Fig. 1.

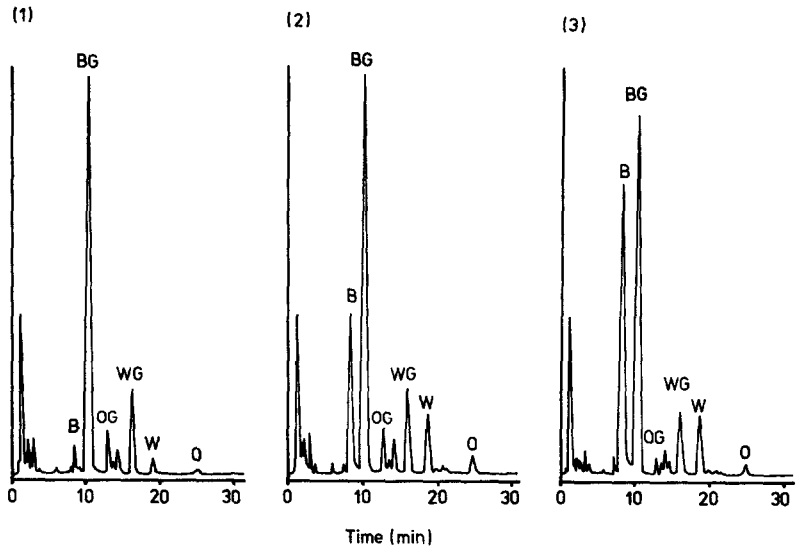

Fig. 8. Chromatograms of Scutellariae Radix. Samples: (1), market sample 1; (2), sample from China (Santo 1); (3), cultivated sample.

To determine the most appropriate extraction time, the sample was refluxed with the mobile phase for 15, 30, 60 and 120 min, and three times for 30 min. The extraction efficiency for each flavonoid was invariant after 30 min (Fig. 7).

From these results, samples were extracted with the mobile phase under reflux for 30 min.

Analytical results

Fig. 8 illustrates the chromatograms obtained using the ion-pair method for Scutellariae Radix purchased from the drug market and from China and for a sample cultivated in our herb garden. Table II shows the analytical results for twelve samples.

TABLE II

FLAVONOID CONTENTS (%) IN SCUTELLARIAE RADIX SAMPLES

Sample		*B*	*BG*	*W*	*WG*	*O*	*OG*
Market	1	0.57	12.09	0.41	2.25	0.27	1.23
	2	1.52	9.67	1.03	1.73	0.31	1.46
	3	0.76	15.23	0.57	2.66	0.13	1.30
China	Santo 1	4.65	10.20	2.59	2.10	0.46	0.93
	Santo 2	3.10	16.63	0.26	1.84	0.08	1.03
	Sansei 1	0.17	9.97	0.38	2.15	0.09	1.26
	Sansei 2	3.75	19.64	0.34	2.88	0.10	1.83
	Kahoku	4.06	15.14	0.39	2.59	0.15	0.64
	Tenshin	0.66	16.25	0.15	3.20	0.07	1.60
	Naimouko	0.54	11.73	0.64	2.03	0.31	1.81
	Kokuryuko	12.89	14.59	0.86	2.73	0.39	1.25
Cultivated sample		11.94	10.61	4.61	2.20	0.28	0.33

The content of baicalin varied from 9.67 to 19.64%, as found previously[13,14]. However, the contents of baicalein were considerably different, between 0.17 and 12.89%; containing more than 10% of baicalein have not previously been reported. Although the other four flavonoids were minor components, they were present in all samples.

CONCLUSIONS

The ion-pair HPLC technique was applied as a new method for the analysis of Scutellariae Radix. TAA as the counter ion influenced the behaviour only of the glucuronides, made it possible to elute them between baicalein and wogonin and therefore baicalein, wogonin, oroxylin-A and their glucuronides could be determined simultaneously. This method is simpler, more rapid and precise than previous methods, having several advantages: (1) it is an isocratic HPLC system; (2) no pretreatment is required except for extraction; (3) the mobile phase has good extraction efficiency and (4) the simultaneous determination of the six flavonoids, for the first time for oroxylin-A and its glucuronide, is possible. The method seems to be useful for the quality estimation of Scutellariae Radix.

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