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RAPID AND SIMPLE DETERMINATION OF SENNOSIDE A AND B IN RHEI RHIZOMA BY ION-PAIR HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

A simple and precise ion-pair high-performance liquid chromatographic method was developed for the determination of sennoside A and B in Rhei Rhizoma. A reversed-phase chromatographic system consisting of a chemically bonded ODS silica gel column with an acetate buffer (pH 5.0)–acetonitrile (68:32), containing 5 m*M* tetra-*n*-heptylammonium bromide, as the mobile phase was used. Sennoside A and B in this crude drug were separated and determined within 20 min after direct injection of the solution extracted with 70% methanol. The results for various samples are presented.

INTRODUCTION

The crude drug Rhei Rhizoma[1] (rhubarb; "Da huang" in Chinese), is well known as a laxative and is frequently used in oriental pharmaceutical preparations. It has been reported that Rhei Rhizoma contains a large number of phenolics, such as dianthrones, anthrones, anthraquinones, stilbenes, chromones, phenylbutanones, naphthalene derivatives and tannins[2]. Sennosides[3–7] are the laxative agents, and the main components are sennoside A and B. Therefore, the quality of Rhei Rhizoma has been evaluated using sennosides as indicator compounds.

Rhei Rhizoma has been examined by spectrophotometry[8,9], polarography[10], paper partition chromatography (PPC)[11] and thin-layer chromatography (TLC)[12–14], mainly for total sennosides, and these methods involve complicated and time-consuming procedures to remove impurities. High-performance liquid chromatography (HPLC) methods for Rhei Rhizoma have also been examined using dimethylamino-bonded[15], phenyl[16] or ODS[17–19] silica gel columns to separate and determine sennoside A and B, but even using these methods sennoside A and B cannot be separated from the other components in the crude drug without pre-treatment[15–18] or application of the gradient technique[19]. On the other hand, simple HPLC methods for the evaluation of senna (leaves and pods) have been established using dimethylamino-bonded[20,21], C_8[22,23] or ODS[24,25] silica gel columns, and they allow the separate determination of sennoside A and B. However, their application to the deter-

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mination of sennoside A and B in Rhei Rhizoma was limited, because this crude drug contains a large number of phenolics in addition to sennosides, compared with senna, and there was interference between sennoside A and B.

Recently, ion-pair chromatography has been applied to the determination of some natural products in plants, *e.g.*, berberine[26], aconitine[27] and ephedrine alkaloids[28] as basic compounds, and glycyrrhizin[29] and baicalin[30] as acidic compounds, because it provides appropriate retentions of complexes formed with the counter ion. Also, ion-pair techniques[22–24] have been used for the determination of sennosides in senna and its pharmaceutical preparations, but they are not applicable to Rhei Rhizoma, especially owing to interference by the sennoside B peak.

In this study, an ion-pair HPLC method was developed for the determination of sennoside A and B in Rhei Rhizoma.

EXPERIMENTAL

Plant materials

Commercial Rhei Rhizoma samples were purchased from Alps Pharmaceutical and *Rheum palmatum* samples were supplied by the Institute of Materia Medica, Chinese Academy of Medical Sciences (Beijing, China).

Apparatus

A Hitachi Model 655 liquid chromatograph equipped with a Hitachi 638 UV spectrophotometer and a stainless-steel column (150 × 4 mm I.D.) packed with chemically bonded ODS silica gel (TSK Gel 120 A, 5 μm; Toyo Soda, Tokyo, Japan) was used. The peak area was counted by a SIC 7000 A integrator.

Reagents

Sennoside A and B were purchased from Sandoz (Basle, Switzerland). Tetramethyl-, tetraethyl-, tetra-*n*-propyl-, tetra-*n*-butyl- and tetra-*n*-amylammonium bromide were purchased from Tokyo Kasei Kogyo (Tokyo, Japan) and tetra-*n*-hexyl- and tetra-*n*-heptylammonium bromide from Aldrich (Milwaukee, WI, U.S.A.). Acetonitrile of chromatographic grade was used.

HPLC conditions

Acetate buffer (pH 5.0)–acetonitrile (68:32) containing 5 m*M* tetra-*n*-heptylammonium bromide was used as the mobile phase. The column temperature was maintained at 50°C and the flow-rate was 1.0 ml/min. The eluted substances were detected by a UV detector operated at a wavelength of 308 nm.

Assay procedure

Dry Rhei Rhizoma powder (1.0 g) was dissolved in 25 ml of 70% methanol, then irradiated supersonically for 30 min, centrifuged at 1600 *g* and decanted. The residue was washed twice with 10-ml portions of 70% methanol. The extracts and washings were placed in a 50-ml volumetric flask and diluted to 50 ml with 70% methanol. A 10-μl volume of this solution was injected into the HPLC system. The contents of sennoside A and B in Rhei Rhizoma were calculated from the relevant peak areas.

Calibration graphs and detection limits

The calibration graphs for sennoside A and B were obtained over the concentration ranges 5.0–500.0 and 5.0–200.0 μg/ml, respectively. The corresponding regression equations were $y = 2700x - 8300$ ($r = 0.999$) and $y = 2800x - 6700$ ($r = 0.999$) and the detection limits were 1.2 and 1.0 ng, respectively, at a signal-to-noise ratio of 3:1 for the peak heights.

RESULTS AND DISCUSSION

As the HPLC determination of sennoside A and B in Rhei Rhizoma using an isocratic system requires pre-treatment of the samples, two HPLC conditions for senna were applied. One was the use of a dimethylamino column with methanol–water–acetic acid (80:20:6.5) as the mobile phase (conditions I), and the other was the use of an ODS column with water–methanol (70:30) containing 5 m*M* tetrabutylammonium (pH 7.5) as the mobile phase (conditions II). Under conditions I, sennoside B was separated clearly from the other components in the crude drug, but the peak shape of sennoside A was slightly broader than that of the sennoside A standard. This suggested that the sennoside A peak contained impurities. In contrast, the elution order of sennoside A and B was reversed under conditions II, and sennoside B could not resolved from the other peaks.

The ion-pair technique has several advantages, *e.g.*, the counter ion only affects the ionic compounds and it is possible to control the retention time by changing the counter ion and its concentration. The pH of the mobile phase also influences the peak retention. Therefore, an ion-pair HPLC method was examined for Rhei Rhizoma.

HPLC conditions

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

Tetra-*n*-butylammonium bromide (TBA) has been used as a counter ion to separate and determine sennoside A and B in senna leaves and preparations[23,24]. Various tetraalkylammonium salts, such as tetramethyl-, tetraethyl-, tetra-*n*-propyl-, tetra-*n*-butyl-, tetra-*n*-amyl-, tetra-*n*-hexyl- and tetra-*n*-heptylammonium bromide, were examined for the elimination of the impurities from sennoside B. Each counter ion was added to the acetate buffer (pH 5.0)–acetonitrile (68:32) mobile phase at a final concentration of 5 m*M*. With counter ions having an alkyl chain shorter than C_4, sennoside A and B were not retained on the column when the mobile phase was water–acetonitrile (68:32). Therefore, longer counter ions were required for the separation of sennoside B from the other components. As shown in Fig. 1, the retention of sennoside A and B increased in proportion to the length of the alkyl chain of the counter ions, and the optimal separation was obtained using tetra-*n*-heptylammonium bromide (THpA).

The THpA concentration in the mobile phase was varied from 0 to 20 m*M*. The retention of sennoside A and B depended on the concentration of THpA, being maximal at 5 m*M* THpA (Fig. 2).

The acetonitrile concentration was varied from 26 to 38%. Sennoside B began

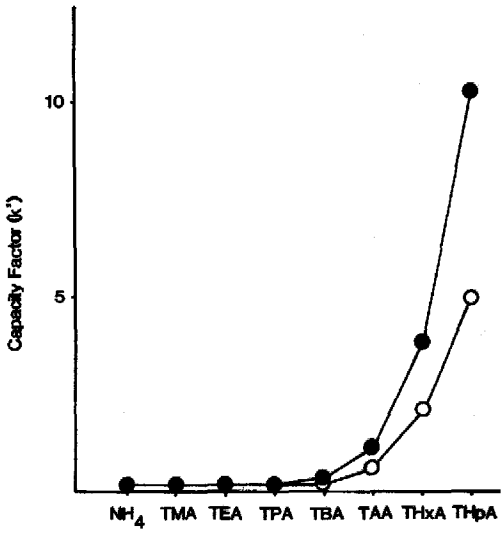

Fig. 1. Effect of various counter ions on capacity factors, k', of sennoside A (●) and sennoside B (○). Counter ions: NH_4^+ = ammonia; TMA = tetramethylammonium bromide; TEA = tetraethylammonium bromide; TPA = tetra-*n*-propylammonium bromide; TBA = tetra-*n*-butylammonium bromide; TAA = tetra-*n*-amylammonium bromide; THxA = tetra-*n*-hexylammonium bromide; THpA = tetra-*n*-heptylammonium bromide. Flow-rate: 1 ml/min. Temperature: 50°C. Mobile phase: acetate buffer (pH 5.0)–acetonitrile (68:32).

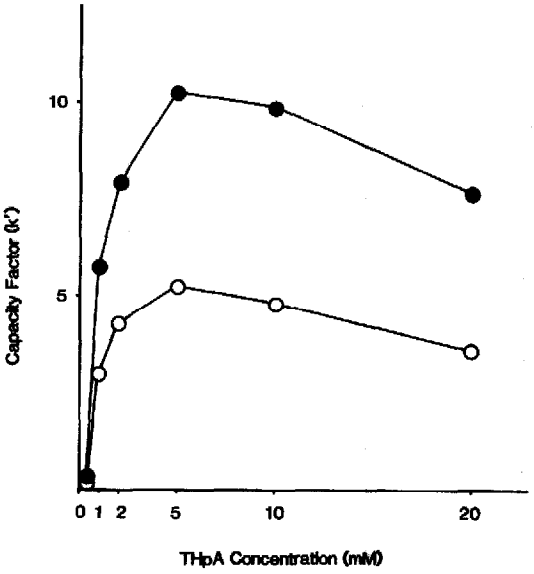

Fig. 2. Effect of THpA concentration on capacity factor, k'. For solutes and chromatographic conditions, see Fig. 1.

to separate from the other peaks in Rhei Rhizoma at an acetonitrile concentration of 32%; therefore, this concentration was selected for subsequent work, based on resolution and retention time (Fig. 3).

The pH of the mobile phase affected the retention times of sennoside A and B. As shown in Fig. 4, below pH 3, where the ion suppression effect is stronger than the ion-pair effect, both sennoside A and B were negligibly retained on the ODS column. THpA affected sennoside A and B, having a carboxylic moiety, above pH 3.5, and the capacity factor increased. The curves of pH *versus* the retention did not show a plateau from around pH 5, where the carboxylic acid function is completely dissociated. Although the maximal retention of sennoside A and B occurred at pH 4.0, the best resolution from other peaks was obtained at pH 5.0.

The column temperature was found to affect the retention time only slightly, and was subsequently kept at 50°C.

Finally, a mobile phase consisting of acetate buffer (pH 5.0)–acetonitrile (68:32) containing 5 m*M* THpA was selected as the optimum for the separation of sennoside A and B from the impurities in Rhei Rhizoma.

Extraction conditions

As sennoside A and B are heat labile, the supersonic irradiation method was used for extraction. Table I shows the extraction efficiency of various solvents, namely phosphate buffer (pH 6.0), 70% methanol, 50% acetone and the present mobile phase. Our previous experiments indicated that a higher extraction efficiency would be obtained by use of a mobile phase containing a counter ion, but the best extraction solvent was found to be 70% methanol.

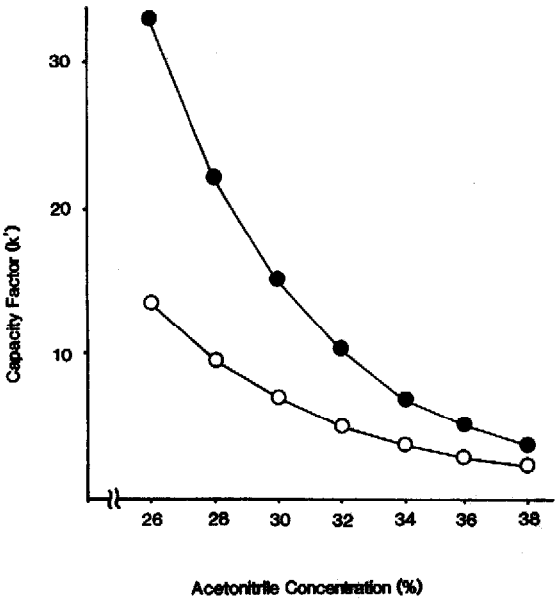

Fig. 3. Effect of acetonitrile concentration on capacity factor, k'. Mobile phase: acetate buffer (pH 5.0)–acetonitrile containing 5 m*M* THpA. Solutes and other conditions as in Fig. 1.

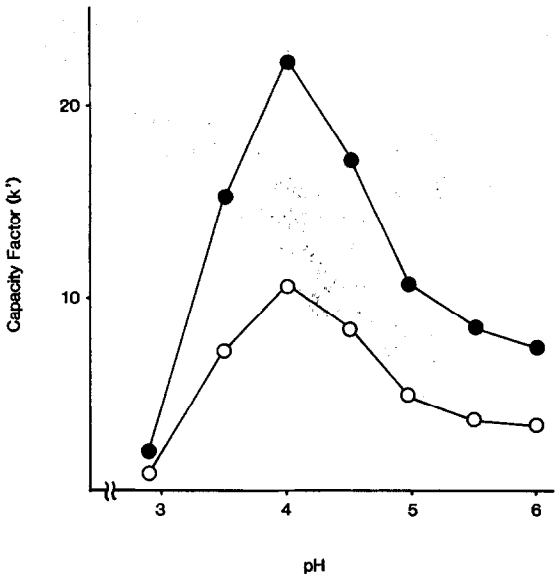

Fig. 4. Effect of pH on capacity factor, k'. Mobile phase: acetate buffer (pH 5.0)–acetonitrile containing 5 m*M* THpA. Solutes and other conditions as in Fig. 1.

From these findings, samples were subsequently extracted with 70% methanol under supersonic irradiation for 30 min.

The values of sennoside A obtained by using a dimethylamino column with a methanol–water–acetic acid (80:20:6.5) mobile phase were compared with those obtained by the proposed ion-pair HPLC method. The values were about 20% higher with the dimethylamino column method. To confirm this difference, the sennoside A peak separated by the dimethylamino column was collected and re-analysed by the ion-pair method. An unknown peak appeared at about 10 min with a peak area about 20% of that for sennoside A. The values for sennoside B were in good agreement with that obtained by the dimethylamino column method.

TABLE I

EFFECT OF EXTRACTION SOLVENT

Solvent	*Sennoside A (%)*	*Sennoside B (%)*
Mobile phase	71.7	93.3
Phosphate buffer (pH 6.09)	77.9	90.2
70% Methanol	100.0	100.0
50% Acetone	96.3	99.9

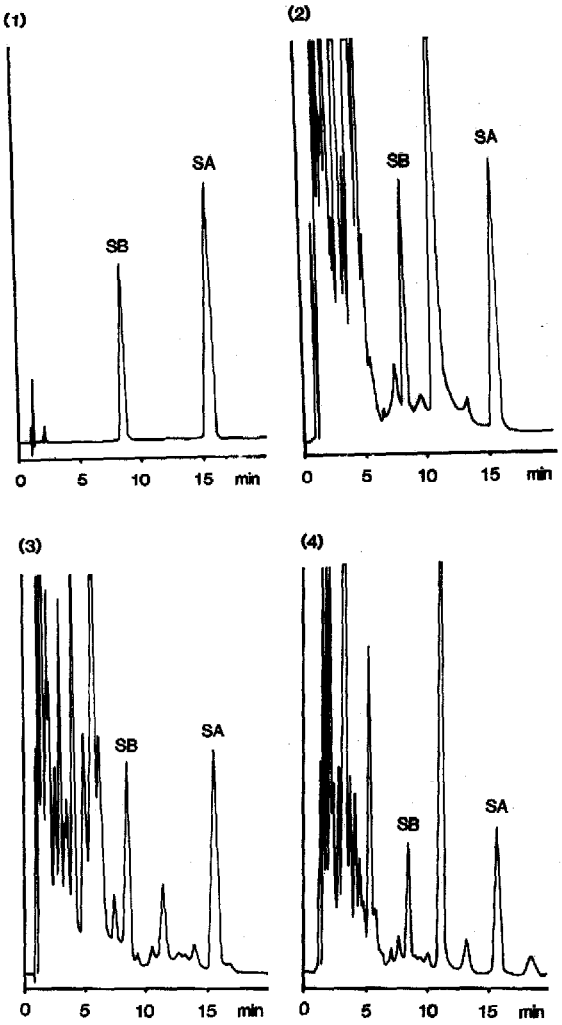

Fig. 5. Chromatograms of standards and Rhei Rhizoma. Samples: (1) standard; (2) market 1; (3) market 7; (4) *Rheum palmatum* 1. Peaks: SA = sennoside A; SB = sennoside B.

Analytical results

Fig. 5 illustrates the chromatograms obtained on applying the ion-pair method to Rhei Rhizoma. Free anthraquinones were considered to be eluted near the sennoside A and B peaks, because the retentions of sennoside A and B were greater on the column due to pairing with THpA than when no counter ion was used. The peak eluted between sennoside A and B was identified as aloe-emodin from the comparison of the retention time with that of a standard. Other free anthraquinons, namely chrisophanol, emodin and rhein, were not eluted under these conditions even after 60 min, so there were no conspicuous peaks after sennoside A. Table II gives the results for ten samples. The contents of sennoside A and B varied from 2.02 to

TABLE II

CONTENTS OF SENNOSIDE A AND B IN RHEI RHIZOMA

Sample	*Sennoside A (mg/g)*	*Sennoside B (mg/g)*	*Sennoside A: sennoside B*
Market 1	11.64 mg/g	5.58 mg/g	1:0.48
Market 2	3.11	0.97	1:0.31
Market 3	11.44	4.65	1:0.41
Market 4	7.05	4.53	1:0.64
Market 5	11.19	5.29	1:0.47
Market 6	6.34	3.27	1:0.52
Market 7	9.16	3.89	1:0.42
Market 8	6.95	3.69	1:0.53
Rheum palmatum 1	6.74	3.17	1:0.47
Rheum palmatum 2	2.02	1.42	1:0.70

11.64% and from 0.87 to 5.58%, respectively, and the ratio of sennoside B to sennoside A varied from 0.31 to 0.70.

CONCLUSIONS

An ion-pair HPLC technique was applied to the determination of sennoside A and B in Rhei Rhizoma. THpA as the counter ion influenced the behaviour of sennoside A and B and permitted them to be eluted separately from other components in Rhei Rhizoma that interfered with sennoside B under previously used ion-pair HPLC conditions.

This method seems simpler and more rapid than previous methods. Its advantages are that (1) it involves an isocratic HPLC system, (2) no pre-treatment is required except for extraction and (3) there are no conspicuous peaks after sennoside A. The method seems to be useful for the qualitative analysis of Rhei Rhizoma.

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