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ORIGINAL PAPER

Simple and rapid analysis of sennoside A and sennoside B contained in crude drugs and crude drug products by solid-phase extraction and high-performance liquid chromatography

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Abstract The sennoside A (SA) and sennoside B (SB) contents of various samples of crude drugs were determined using solid-phase extraction (SPE) and HPLC. The samples examined were crude drugs (senna leaf, senna pods, and rhubarb), conventional crude drug products, and Kampo formulations. The sample solution was purified using an Oasis MAX cartridge, which has strong anion-exchange and reversed-phase properties. The samples containing SA and SB were dissolved in a solution of methanol–0.2% sodium bicarbonate (7:3, v/v) and applied to the Oasis MAX cartridge. The cartridge was washed with a solution of methanol containing 1% acetic acid. SA and SB were eluted with methanol–water–formic acid (70:30:2, v/v), and the eluate was used as the sample solution for HPLC analysis. SA and SB were analyzed using a conventional octadecylsilyl (ODS) column at a detection wavelength of 380 nm; water–acetonitrile–phosphoric acid (800:200:1, v/v) was used as the mobile phase. The SA and SB components in most samples were completely separated from other interfering constituents within 10 min. In particular, several interfering peaks adjacent to the SB peak were eliminated by SPE using the Oasis MAX cartridge. On subjecting the Kampo extracts to an additional recovery experiment, high recovery rates of SA and SB were obtained. The method employed in this study proved to be a simple and rapid method for the quantification of SA and SB.

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Introduction

Senna leaf and pods [*Cassia angustifolia* or *C. acutifolia* (Leguminosae)] and rhubarb [*Rheum palmatum*, *R. tanguticim*, *R. officinale*, or *R. coreanum* (Polygonaceae)] are well-known laxatives that are widely used over-the-counter (OTC) crude drug products. Rhubarb is frequently used in laxative formulas in Kampo medicine. The main purgatives in these crude drugs are sennosides, which are rheindianthrone glucosides, such as sennoside A (SA) and sennoside B (SB) (Fig. 1). SA and SB are stereoisomers, i.e., their asymmetric carbon atoms are at the 10 and 10′ positions, respectively. SA and SB are acidic compounds with 2 carboxylic acid groups; they are insoluble in water and barely soluble in methanol, but are easily soluble in an aqueous solution of methanol (optimum concentration, 30% w/w of water). Furthermore, they are soluble in aqueous solutions of sodium bicarbonate [1].

Many studies have reported the quantitative analysis of SA and SB by isocratic HPLC [2, 3], gradient HPLC [4], ion-pair HPLC [5–7], and capillary electrophoresis [8, 9]. Some of these studies used solid-phase extraction (SPE) to remove the interfering components. Shokawa et al. analyzed the sennoside contents of Senna and Rhubarb using the following two methods: direct ion-pair HPLC analysis without pretreatment of the sample, and ion-suppression HPLC analysis after the treatment of samples on Sep-Pak QMA with an anion-exchange resin. The chromatogram obtained in the second method showed that the SB present in senna could not be completely separated from other interfering components [10]. Moreover, Kondo analyzed

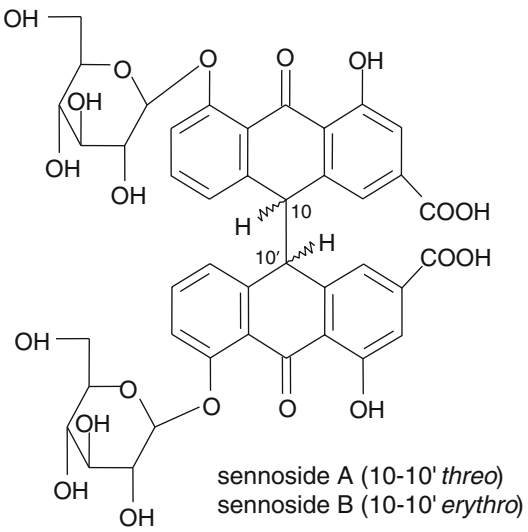

Fig. 1 Structures of sennoside A (SA) and sennoside B (SB)

several Kampo formulations containing rhubarb by HPLC after SPE with DEAE Cellulofine, which is an anion-exchange resin [11]. However, the pretreatment procedure is complicated in this method, and the recovery rate of sennosides is low. Moreover, it is difficult to eliminate the interfering peaks from various crude drugs present in the conventional crude drug products or Kampo extract formulations.

In the present study, we investigated the efficiency of a rather simple and rapid method for the simultaneous analysis of SA and SB in crude drugs (senna leaf, senna pods, and rhubarb), conventional crude drug products, and Kampo formulations. We used an SPE method and HPLC–photodiode array (PDA) detection without an ion-pair reagent.

Materials and methods

Materials

Senna leaf [*Cassia angustifolia*; Japanese Pharmacopoeia, 15th edition (JP15)] was purchased from Mikuni & Co. Ltd. (Osaka, Japan) in 2005. The voucher specimen (No. B010) was deposited in our laboratory. Senna pods (*Cassia angustifolia*; non-Japanese crude drug standard) were purchased from Tochimoto Tenkaido Co. Ltd. (Osaka, Japan) in 2002, and the voucher specimen (No. C300697) was deposited in our laboratory. Further, rhubarb (*Rheum palmatum*; JP15, Gao) was purchased from Tochimoto Tenkaido Co. Ltd. (Osaka, Japan) in 2004, and the voucher specimen (No. A246) was deposited in our laboratory. Three groups of rhubarb (Kinmon-daio, *R. tanguticum*; Gao, *R. palmatum*; Hokkai-daio, *R. palmatum*) were obtained from the Faculty of Pharmaceutical Sciences, Osaka University, in 1985. These samples were identified by Dr. Kaisuke Yoneda, an associate professor at Osaka University. Voucher specimens of the abovementioned groups of rhubarbs (Kinmon-daio, No. I–XI; Gao, No. XII–XXI; Hokkai-daio, No. XXII–XXXIV) were deposited in our laboratory [12].

Several commercially available laxatives containing senna leaf and/or rhubarb and OTC crude drug products were purchased from a pharmacy in Osaka. Powdered conventional crude drug products, their components, and the quantities in which they are consumed per day were as follows. Sample A (tablet): aloe (0.1), glycyrrhiza (0.15), immature orange (0.05), magnolia bark (0.075), paeony root (0.2), rhubarb (0.3), senna leaf (0.7), and zedoary (0.075). Sample B (pill): rhubarb (0.3), paeony root (0.2), cinnamon bark (0.1), calumba (0.1), coptis rhizome (0.15), smilax rhizome (0.15), akebia stem (0.15), ginseng (0.3), glycyrrhiza (0.4), phellodendron bark (0.5), sophora flower (1.0), scutellaria root (0.6), forsythia fruit (0.6), sophora root (0.8), areca (0.6), gardenia fruit (0.5), and chaulmoogra (0.1). Sample C (tablet): senna leaf (0.9) and rhubarb (0.45). Sample D (tablet): senna leaf (1.2) and aloe (0.35). Some vehicles were prescribed for the administration of samples C and D.

We collected several purgatives containing senna leaf and/or rhubarb along with some of the following active ingredients: magnesium carbonate, magnesium hydroxide, magnesium oxide, magnesium sulfate, sodium sulfate, carboxymethyl cellulose sodium salt, and dehydrocholic acid (Wako Pure Chemical Industries Inc., Osaka, Japan), and bisacodyl (Sigma–Aldrich Corporation, St. Louis, MO, USA). Aloe, rose fruit, and pharbitis seeds were purchased from Mikuni & Co. Ltd. [13]. Each of the blank samples of OTC (without SA and SB) was prepared at our laboratory by mixing the raw materials, i.e., prescription drugs without rhubarb and/or senna leaf. The raw materials for the OTC blank samples were purchased from Mikuni & Co. Ltd. and Tochimoto Tenkaido Co. Ltd.

Raw materials containing rhubarb required for the preparation of extracts of 16 Kampo prescriptions (Bofutsushosan, Choijokito, Daijokito, Daiobotanpito, Daiokanzoto, Daisaikoto, Inchinkoto, Jizusoippo, Junchoto, Keishikashakuyakudaioto, Mashiningan, Otsujito, Saikokaryukotsuboreito, San'oshashinto, Tokakujokito, and Tsudosan) were also purchased from Mikuni & Co. Ltd. and Tochimoto Tenkaido Co. Ltd.

Chemicals and solid phase

Standards: SA and SB standards for crude drug testing were purchased from Wako Pure Chemical Industries Ltd. (Cas No. 81-27-6 and 128-57-4, respectively; Osaka,

Japan). The purity of the SA and SB standards was more than 99.0%.

Solid phase: We purchased Oasis MAX (Waters Co. Ltd., 60 mg/3 cc, 30 μm), which has strong anion-exchange and reversed-phase properties. This solid phase is a poly (divinylbenzene-co–*N*-vinylpyrrolidone) copolymer that contains 0.25 meq/g of quaternary amine groups.

Preparation of samples

Preparation of blank samples of conventional crude drug products

Conventional crude drug products of a commercial laxative that did not contain senna leaf or rhubarb were manufactured for the purpose of this study.

Preparation of Kampo extracts

Sixteen Kampo preparations containing rhubarb were manufactured for the purpose of this study. The dose per day (in grams) of each crude drug was measured according to the prescribed dose of Kampo formulations manufactured by Tsumura & Co. (only Saikokaryukotsuboreito was manufactured by Kracie Pharma Ltd.). Water (300 ml) was added to these crude drug mixtures, and the solution was heated for 1 h and filtered through gauze. After cooling, the extract was centrifuged for 5 min at 2,000×*g*. The supernatant was collected and freeze-dried to obtain the powder. Blank samples of Kampo formulations without rhubarb were also manufactured.

Extraction and solid-phase treatment

Twenty-five milliliters of a solution of methanol–0.2% sodium bicarbonate (7:3, v/v) were added to 1.0 g of the powdered sample of each crude drug or crude drug product and shaken for 10 min. After centrifugation for 5 min at 2,000×*g*, the supernatant was removed. The residue was extracted with 20 ml solution of methanol–0.2% sodium bicarbonate (7:3, v/v). The supernatant was collected, and the volume was adjusted to 50 ml with methanol.

Two milliliters of the supernatant containing methanol was directly transferred onto an Oasis MAX cartridge, which was conditioned with 2 ml of methanol and 2 ml of 0.2% sodium bicarbonate. The cartridge was first washed with 2 ml of water, followed by 2 ml of methanol, and finally with 5 ml of methanol containing 1% acetic acid. SA and SB were eluted with methanol–water–formic acid (70:30:2, v/v). The volumes of the eluted fractions were adjusted to 2 ml and used as sample solutions in HPLC.

Apparatus and HPLC conditions

The HPLC instrument consisted of a Shimadzu LC-SPD10AD$_{VP}$ system (Shimadzu Co., Kyoto, Japan). The HPLC conditions were as follows: wavelength for quantitative analysis, 380 nm; column properties, TSKgel ODS-80Ts-QA, 4.6 mm (ID) × 150 mm, 5 μm (Tosoh, Tokyo, Japan); mobile phase, water–acetonitrile–phosphoric acid (800:200:1, v/v); column temperature, 40°C; flow rate, 1.0 ml/min (retention time of SA was approximately 10 min); injection volume, 20 μl.

Calibration curve

The SA and SB standard mixture (10 mg) dissolved in 50 ml of 0.1% sodium bicarbonate (i.e., the concentration of both SA and SB was 200 μg/ml) was used as the stock solution. The stock solution was adequately diluted with methanol (0.02–100 μg/ml) to obtain the calibration curve. The peak area was plotted against the amount of SA and SB. The standard calibration curves were subjected to linear regression analysis.

Recovery test for SA and SB added to the Kampo preparations

The recovery tests were conducted at two concentrations of SA + SB—500 and 50 μg/g. SA and SB at 250 μg/g each and SA + SB at 25 μg/g were added to the blank sample, which contained Kampo extracts without the rhubarb. Sample solutions were prepared by SPE with Oasis MAX, and the recovery rates of SA and SB were determined.

Results and discussion

HPLC analysis

SA and SB were analyzed using a conventional octadecylsilyl (ODS) column and an ion-suppression mobile phase; they were simultaneously analyzed at 380 nm. The correlation coefficient for SA and SB was excellent (average, 0.999), and it covered a wide range (0.02–100 μg/ml). The detection limit for SA and SB in the sample solution was 0.01 μg/ml (S/N = 5) (0.5 μg/g of each, SA and SB, was present in the sample).

Extraction and SPE of SA and SB

SA and SB are acidic compounds (pKa = 4.0) that have two carboxylic acid groups. Sennosides are highly soluble in aqueous solutions of sodium bicarbonate–methanol. These sennosides dissociate completely in a solution of

0.2% bicarbonate–methanol (pH 7.5–8.5), and this enables their binding to the Oasis MAX resin. After the sennoside solution was applied to Oasis MAX, the cartridge was washed with 2 ml of water and 2 ml of methanol to eliminate neutral or basic compounds. The cartridge was washed with a solution of acetic acid in methanol before the elution of SA and SB with methanol–water–formic acid (70:30:2, v/v), ensuring that acidic compounds which interfere with the detection of SA and SB in HPLC analysis are effectively eliminated. An interfering peak adjacent to the SB peak was observed in the chromatogram when the cartridge was washed with a solution of formic acid and methanol instead of acetic acid and methanol. Therefore, in this experiment, the cartridge was washed with a 5 ml solution of methanol and 1% acetic acid before SA and SB. Under this condition, SA and SB were still retained in Oasis MAX. Subsequently, methanol–water–formic acid (70:30:2, v/v) was used to elute SA and SB from Oasis MAX. Using this procedure that utilizes Oasis MAX, the interfering peak adjacent to the SB peak in the chromatogram was efficiently eliminated.

Analysis of sennosides in crude drug preparations

Three crude drugs containing sennosides extracted from senna leaf, senna pods, and rhubarb were applied to the Oasis MAX cartridge to determine their SA and SB contents. The chromatograms of these three crude drugs are shown in Fig. 2. The chromatogram of the senna leaf extract that was not treated with SPE (Fig. 2A) showed numerous interfering peaks adjacent to the SB peak. The SB peak was obscured by a large interfering peak. Therefore, quantitative analysis of SA and SB was very difficult. However, these interfering constituents were eliminated by treatment with the Oasis MAX cartridge (Fig. 2a). In addition, as shown by the chromatogram of the extracts of senna pods (Fig. 2B), many interfering peaks were observed adjacent to the SB peak, similar to those observed for senna leaf extracts; these peaks were eliminated by the abovementioned treatment (Fig. 2b). The chromatogram for the rhubarb extract showed small interfering peaks close to the SA peak (Fig. 2C); these were also eliminated by treatment with the Oasis MAX cartridge (Fig. 2c). The

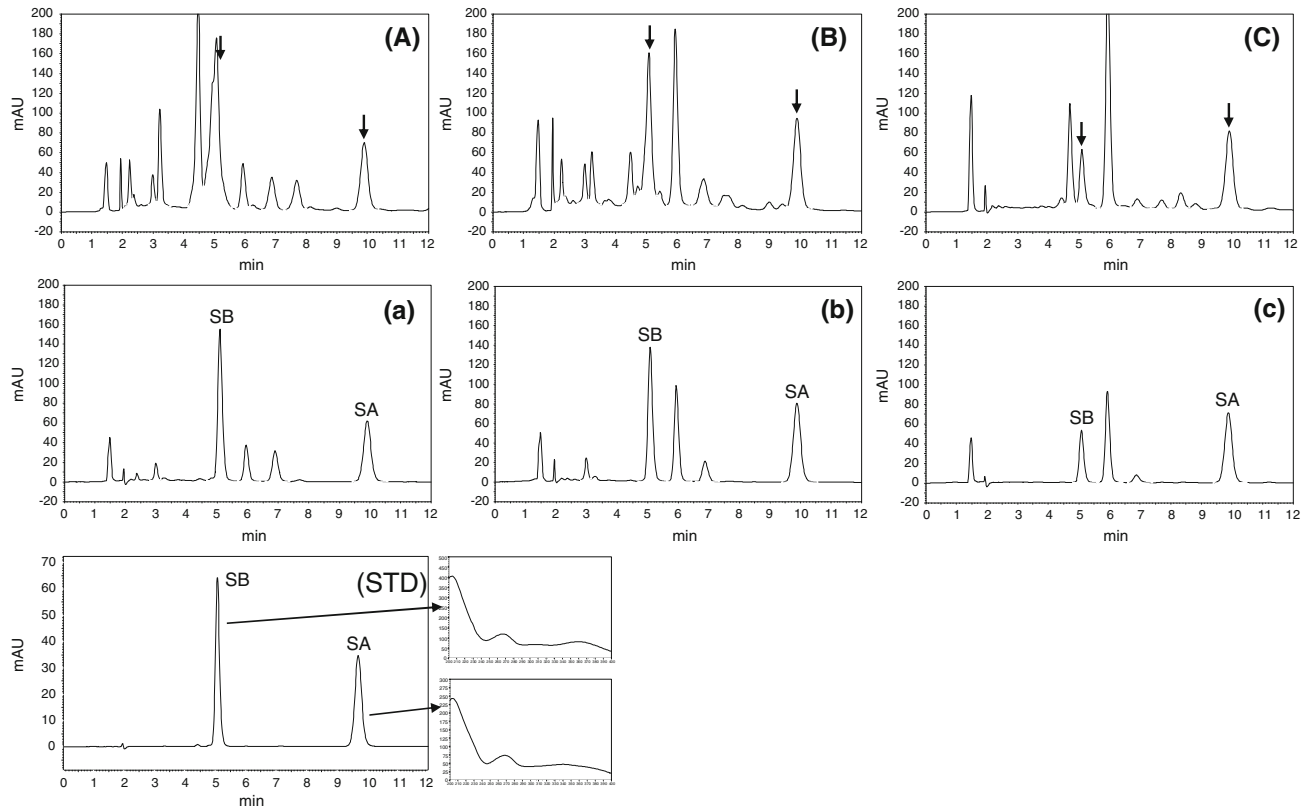

Fig. 2 HPLC profiles of crude drugs containing sennosides. Chromatograms of the crude extract of **A** senna leaf, **B** senna pods, and **C** rhubarb. Chromatograms of the sample solution (applied to the Oasis MAX cartridge) of **a** senna leaf, **b** senna pods, and **c** rhubarb. (STD) Chromatograms and spectra of SA and SB. A standard mixture of SA and SB, 50 μg/ml each, was applied to the column. SA and SB were identified by comparing the retention times and UV spectra recorded with the HPLC–photodiode array (PDA) detection method (200–400 nm) to standard reference data

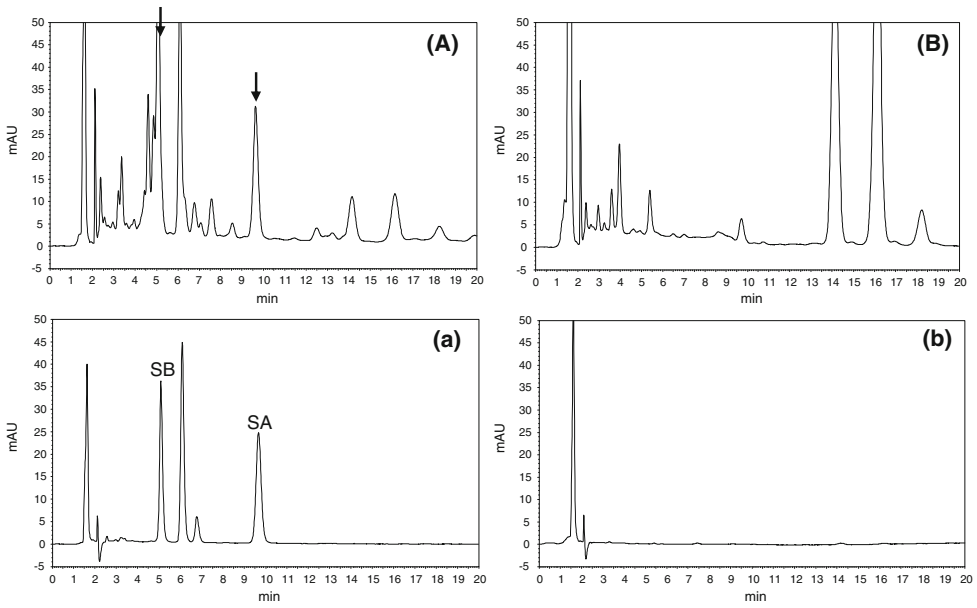

Fig. 3 HPLC profiles of an over-the-counter (OTC) laxative containing senna leaf and rhubarb (sample A). Chromatograms of: **A** a crude extract of the laxative; **a** a sample solution (applied to the Oasis MAX cartridge) of the laxative; **B** a crude extract of the laxative without senna leaf and rhubarb (blank); **b** a sample solution (applied to the Oasis MAX cartridge) of the laxative without senna leaf and rhubarb (blank)

SA and SB content in senna leaf was 1.12%; senna pods, 1.24%; and rhubarb, 0.53%. The SA:SB ratio in senna leaf was 2:3; senna pods, 1:1; and rhubarb, 2:1. This clean-up effect of the above SPE treatment was also confirmed for the other rhubarbs: Gao (*R. palmatum*), Kinmon-daio (*R. tanguticum*), and Hokkai-daio (*R. palmatum*).

Analysis of the sennosides present in conventional crude drug products

Senna leaf, senna pods, and rhubarb are widely used in medicine, together with other crude drugs such as aloe, bisacodyl, or salt-based laxatives, because of their laxative properties. Therefore, many typical OTC drugs and drugs containing rhubarb and senna leaf that are prescribed as laxatives were subjected to SPE and analyzed by HPLC. The chromatograms thus obtained are shown in Fig. 3.

A crude extract of one of the prescribed laxatives containing eight crude drugs (sample A) was applied to the Oasis MAX cartridge, and all of the interfering constituents were eliminated. The chromatogram of the crude extract is shown in Fig. 3A, and the chromatogram obtained after SPE treatment of the crude extract is shown in Fig. 3a. Blank samples of the laxative (removed senna leaf and rhubarb from sample A) were prepared. The chromatograms of the blank samples also showed interfering peaks adjacent to the retention times of SA and SB peaks (Fig. 3B). These peaks were completely eliminated by the abovementioned treatment (Fig. 3b). This result suggests that some peaks in Fig. 3a originated from senna leaf or rhubarb. In addition, other commercial drug samples (B, C, and D) that are prescribed as laxatives and contain senna leaf and/or rhubarb were studied. On Oasis MAX treatment, eluted fractions of these samples showed clear SA and SB peaks on the chromatograms, similar to the peaks observed for sample A.

Furthermore, we also examined crude drugs such as aloe, rose fruit and pharbitis seeds, and chemical components such as bisacodyl and magnesium oxide as listed in the "Materials" section, which are known to be active ingredients in purgatives [13]. The abovementioned SPE treatment enabled complete elimination of the interfering peaks at the respective retention times of SA and SB. This indicated complete elimination of the interfering constituents.

Analysis of the sennosides in Kampo extracts

The efficiency of SPE treatment of the Kampo prescriptions manufactured for this study was examined. Each of the 16 Kampo prescriptions containing rhubarb was applied to the Oasis MAX cartridge and analyzed with HPLC. Most of the interfering peaks obtained on chromatograms of all of the Kampo prescriptions were eliminated, and fine SA and SB peaks were observed.

The chromatogram of crude extracts of Bofutsushosan, which contained 18 crude drugs, showed many interfering peaks adjacent to the SA and SB peaks, and analysis of SB was rather difficult because the SB peak was obscured by the interfering peaks (Fig. 4A). Therefore, the crude extract was applied to the Oasis MAX cartridge. After these peaks had been eliminated, the typical SA and SB peaks could be distinguished (Fig. 4a). In addition, the chromatogram of a blank sample, in which the peaks of rhubarb were eliminated, showed interfering peaks at the retention times of SA and SB (Fig. 4B). These peaks were almost completely eliminated by the abovementioned treatment (Fig. 4b).

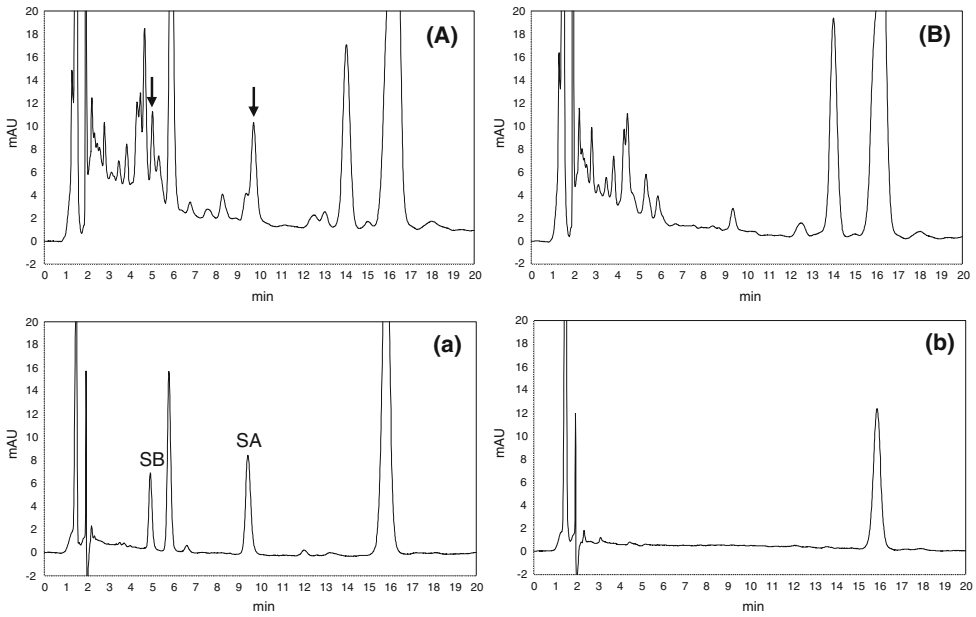

Fig. 4 HPLC profiles of Bofutsushosan. Chromatograms of: **A** a crude extract of Bofutsushosan; **a** a sample solution (applied to the Oasis MAX cartridge) of Bofutsushosan; **B** a crude extract of Bofutsushosan without the rhubarb (blank); **b** a sample solution (applied to the Oasis MAX cartridge) of Bofutsushosan without the rhubarb (blank)

For the prescribed Kampo formulations containing many crude drugs, it was important to determine the wavelengths of the components for quantitative analysis. The extracts present in Bofutsushosan were subjected to solid-phase treatment using Oasis MAX, and their spectra were compared. Even though interfering peaks were observed adjacent to the SB peak at 280 and 340 nm, they were not detected at 380 nm. Further, a chromatogram with distinct SA and SB peaks was obtained after SPE treatment using Oasis MAX.

Additional recovery test for SA and SB

Recovery tests were conducted for the Kampo formulations listed in Table 1. Two concentrations of SA + SB—500 and 50 μg/g (each 250 μg/g or 25 μg/g of SA and SB) were added to 1 g of blank sample, which was manufactured from Kampo formulations that did not contain rhubarb. When SPE treatment of the Kampo formulations using Oasis MAX was performed, the recovery rates after the addition of SA + SB at concentrations of 500 and 50 μg/g were 98–100% and 94–104%, respectively. The recovery rates were similar for all of the samples.

Table 1 Recoveries of sennosides in Kampo extracts

Formula name[a]	Added			
	250 (μg/g)[b]		25 (μg/g)[b]	
	Recovery (%)[c]	C.V. (%)[d]	Recovery (%)[c]	C.V. (%)[d]
Bofutsushosan	98.4	0.8	97.2	1.1
Choijokito	98.6	1.7	93.8	1.6
Daijokito	98.6	0.6	94.3	1.7
Daiobotanpito	100.3	1.0	99.4	1.2
Daiokanzoto	97.6	1.9	96.2	2.4
Daisaikoto	98.9	1.7	96.2	2.6
Inchinkoto	98.7	1.9	97.3	1.6
Jizusoippo	101.4	0.8	104.2	0.8
Junchoto	100.2	0.9	102.4	1.7
Keishikashakuyakudaioto	98.8	1.4	101.3	2.4
Mashiningan	98.6	1.6	102.3	2.1
Otsujito	99.3	1.4	102.2	1.6
Saikokaryukotsuboreito	100.3	0.8	99.7	0.8
San'oshashinto	98.8	1.2	96.6	1.4
Tokakujokito	98.1	1.6	97.2	1.5
Tsudosan	97.8	2.1	95.3	1.8

[a] Each privately manufactured Kampo extract eliminating rhubarb was prepared according to "Preparation of Kampo extracts" and "Extraction and solid-phase treatment" in "Materials and methods"

[b] Each 250 or 25 μg/g of SA and SB was spiked, respectively

[c] Recovery data represent the total recoveries of SA and SB

[d] $n = 3$

Conclusion

We have developed a simple and rapid analytical method involving SPE for the detection of sennosides present in crude drugs, crude drug products, and extracts of Kampo formulations. The application of an SPE procedure using the Oasis MAX cartridge, which has strong anion-exchange and reversed-phase properties, enabled the strong adsorption of acidic substances such as sennosides. After the cartridge was washed with methanol containing 1% acetic acid, the SA and SB that adhered to the Oasis MAX

resins were easily eluted by the addition of methanol–water–formic acid (70:30:2, v/v). Furthermore, on the basis of the spectra obtained at 380 nm, we concluded that almost all of the interfering peaks adjacent to the SA and SB peaks on the chromatograms were eliminated. The method employed in this study, wherein we used a conventional column and a solution of water–acetonitrile–phosphoric acid (simple mobile phase) without an ion-pair reagent, enabled rapid and easy baseline separation of both SA and SB from the other components contained in crude drugs, crude drug products, and extracts of Kampo formulations.

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