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Simultaneous determination of ephedrine, pseudoephedrine, norephedrine and methylephedrine in Kampo medicines by high-performance liquid chromatography

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Abstract

A simultaneous high-performance liquid chromatographic method for the determination of ephedrine, pseudoephedrine, norephedrine and methylephedrine (ephedrine alkaloids) in Kampo medicines which contain *Ephedrae Herba* was established. The analysis can be accomplished within 25 min with a Wakosil-II 5C18 HG column by isocratic elution using a mixture of water, acetonitrile and sodium dodecyl sulfate (65:35:0.4) as the mobile phase at a flow-rate of 1.0 ml min^{-1}, and detection at 210 nm. The detection limits of ephedrine alkaloids are 0.37–1.06 μM per injection (5 μl). This method was applied to analyze the quantities in eight Kampo decoctions; Mao-to, Makyo-yokukan-to, Makyo-kanseki-to, Yokuinin-to, Sho-seiryu-to, Keima-kakuhan-to, Kakkon-to and Kakkon-to-ka-senkyu-sin'i. The concentration (per *Ephedrae Herba* gram) of ephedrine alkaloids was higher in the Makyo-kanseki-to decoction than in the others. Calcium sulfate from *Gypsum Fibrosum* raised ephedrine alkaloids dissolution in the Makyo-kanseki-to decoction.

Keywords: Kampo medicine; Ephedrine; Pseudoephedrine; Norephedrine; Methylephedrine

1. Introduction

Many kinds of traditional oriental medicines (Kampo medicine in Japan) have been used for treatment of a variety of diseases. The herbal medicine (crude drug) *Ephedrae Herba* contains ephedrine, pseudoephedrine, norephedrine and methylephedrine (ephedrine alkaloids), and is widely prescribed in various Kampo medicines. The Kampo medicines containing *Ephedrae Herba*, such as Mao-to, Makyo-yokukan-to, Makyo-kanseki-to, Yokuinin-to, Sho-seiryu-to, Keima-kakuhan-to, Kakkon-to and Kakkon-to-ka-senkyu-sin'i, are usually called Mao-drugs in

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Japan. These drugs are in strong demand. Mao-to, Kakkon-to and Keima-kakuhan-to are used to improve some syndromes that accompany cold virus infection. Makyo-kanseki-to and Sho-seiryu-to are used clinically as cough remedies. Recently, the effectiveness of Sho-seiryu-to for allergic nasal inflammation and bronchial asthma treatment has been recognized by double-blind trial [1]. Kakkon-to-ka-senkyu-sin'i has been used as a sinusitis remedy. Yokuinin-to and Makyo-yokukan-to are often used to treat arthralgia. Ephedrine alkaloids play an important role in the effects of these Kampo medicines. (Fig. 1)

Table 1 shows the combination of crude drug to establish Mao-drugs in which both *Ephedrae Herba* and *Glycyrrhizae Radix* are included. In our previous study on the high-performance liquid chromatography (HPLC) determination of puerarin, daidzin, paeoniflorin, liquiritin, cinnamic acid, cinnamaldehyde and glycyrrhizin in Mao-drug [2], we found that low pH due to organic acids from *Schisandrae Fructus* inhibited glycyrrhizin dissolution in Sho-seiryu-to. In the paper, we could not analyze ephedrine alkaloids simultaneously with other components because we used a reversed-phase column without an ion-pairing reagent. We could separate ephedrine alkaloids in *Ephedrae Herba* only by an ion-pair HPLC [3–6].

In the present study, we improved the method using a mixture of water, acetonitrile and sodium dodecyl sulfate (65:35:0.4) as the mobile phase and applied an ion-pair HPLC to the determination of ephedrine alkaloids in eight Kampo decoctions. The present experiment was performed to elucidate the constituents influencing ephedrine alkaloids content in the decoctions of Mao-drug by HPLC.

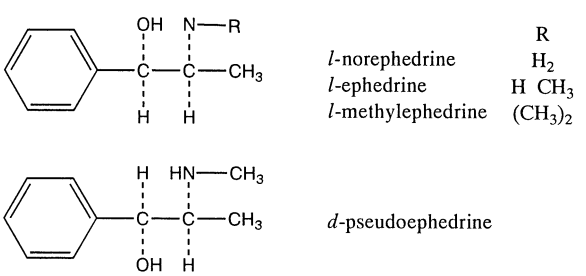

Fig. 1. Structures of ephedrine alkaloids.

2. Experimental

2.1. Materials

Kampo medicines were prepared according to the literature [7] (Table 1). Crude drugs were purchased from Tochimototenkaido (Osaka, Japan). Ephedrine hydrochloride and methylephedrine hydrochloride were purchased from Maruishi Co. (Tokyo, Japan) and norephedrine from Tokyo Kasei Kogyo Co. (Tokyo, Japan). Pseudoephedrine, isolated from *Ephedrae Herba* by a conventional method, was purified by repeated recrystallization from ether [8]. Reagent-grade chemicals and high-purity solvents were used, except when specified otherwise. Ultrapure distilled water with a resistivity greater than 18 MΩ was prepared with deionized-distilled water. Acetonitrile (HPLC grade) was purchased from Wako (Osaka, Japan) and sodium dodecyl sulfate (SDS: sodium lauryl sulfate) from Nacalai Tesque (Kyoto, Japan). Millipore syringe filters (Millex-GP, 0.22 μm pore size) were purchased from Nihon Millipore (Tokyo, Japan).

2.2. Instrumentation and HPLC analysis

The HPLC system consisted of two Tosoh (Tokyo, Japan) CCPD pumps equipped with a Tosoh CCP controller connected to a dynamic mixer, a Tosoh SD-8012 and a Tosoh UV-8000 UV-Vis detector set at 210 nm. Samples were injected with a Model 7125 (Rheodyne, Cotati, CA, USA) sample valve equipped with a 5-μl loop. A SIC Chromatocorder-12 integrator (System Instrument, Tokyo, Japan) was used for data acquisition and integration. Separations were carried out with a Wako Wakosil-II 5C18 HG reversed-phase column (particle size of the packing 5 μm, 150 × 4.6 mm I.D.). Five microliters were injected into the HPLC system.

The mobile phase consisted of a mixture of water–acetonitrile (65:35) containing 0.4% SDS and degassed with an ultrasonic bath prior to use.

Table 1
Combinations of crude drugs in Mao-drug

Crude drug	Crude drugs (g)							
	Mao-to	Makyo-kanseki-to	Makyo-yokukan-to	Yokuinin-to	Sho-seiryu-to	Keima-kakuhan-to	Kakkon-to	Kakkon-to-ka-senkyu-sin'i
Ephedrae Herba	4	4	4	4	3	2	4	4
Glycyrrhizae Radix	1.5	2	2	2	3	2	2	2
Cinnamomi Cortex	3			3	3	3.5	3	2
Paeoniae Radix				3	3	2	3	2
Armeniacae Semen	4	4	3			2.5		
Zingiberis Rhizoma						2	1	1
Zingiberis Siccatum Rhizoma					3			
Zizyphi Fructus						2	4	3
Puerariae Radix							8	4
Coicis Semen			10	8				
Asiasari Radix					3			
Schisandrae Fructus					3			
Pinelliae Tuber					6			
Angelicae Radix				4				
Atractylodis Rhizoma				4				
Cnidii Rhizoma								3
Magnoliae Flos								3
Gypsum Fibrosum		10						

Chromatography was performed at 55°C with a flow-rate of 1.0 ml min^{-1}. The identification and the purity of the chromatographic peaks were estimated using a Model 991J photodiode-array detector (Waters, Milford, MA, USA).

2.3. Standard curve preparation

Ephedrine alkaloids, ephedrine, pseudoephedrine, norephedrine and methylephedrine, were weighed and dissolved in methanol to give serial concentrations within the range 0.18–340 μg ml^{-1}. Each standard sample was prepared in triplicate for ten different concentrations. The standard curve was analyzed using the linear least-squares regression equation derived from the peak area. Concentrations of ephedrine alkaloids in samples were calculated from this regression analysis.

2.4. Preparation of sample solutions

A daily dosage of crude drugs compounded according to each Kampo medicine was placed in a 1-l beaker and boiled with 500 ml water on an electric heater for more than 1.5 h, halving the original volume. The decoction was filtered through a colander while hot, and the volume adjusted to 250 ml with water after cooling. The adjusted decoction was centrifuged ($1500 \times g$ for 10 min) and the supernatant was filtered through a Millipore syringe filter unit and analyzed as the decoction. The crude drug residue after extraction with boiling water was extracted again with boiling methanol (150 ml) for 1 h, and the volume adjusted to 200 ml with methanol after cooling. The adjusted extract was filtered through a Millipore syringe filter unit and analyzed as the residue.

2.5. Interference trial

A daily dosage of crude drugs compounded according to each Kampo medicine without *Ephedrae Herba* was placed in a 1-l beaker and boiled with 500 ml water on an electric heater for more than 35 min, halving the original volume. The decoction was filtered through a colander while hot, and the volume adjusted to 250 ml with water after cooling and centrifugation. The supernatant was filtered through a Millipore syringe filter unit and used for analysis of blank decoction.

2.6. Solutions for recovery study

Recoveries of ephedrine, pseudoephedrine, norephedrine and methylephedrine added to decoctions of Mao-to and Sho-seiryu-to were calculated by comparing three sets of chromatograms: standard solution, control decoctions and spiked decoctions. Standard solutions were prepared by diluting measured volumes of working standards with methanol to a known final volume. Decoctions were divided into five portions, one control and four spiked solutions. The control decoction was prepared by mixing control solution (1 ml) with methanol (1 ml). The spiked decoctions were prepared by adding the four different concentrations of standard solutions (1 ml) to the spiked solution (1 ml). All samples were filtered through a Millipore syringe filter unit and analyzed by HPLC to calculate recovery.

3. Results and discussion

Ion-pair reversed-phase HPLC is often used for the analysis of alkaloids [9–11] in crude drugs and Kampo medicines. The addition of an ion-pairing agent such as SDS, to the mobile phase clearly affects their elution profiles on reversed-phase chromatography. A column of the literature [3] which analyzed Kampo medicines was not available currently, and the most suitable separation condition was not provided in the condition that phosphoric acid was added. Therefore we analyzed Kampo medicines with a Wakosil-II 5C18 HG column by isocratic elution using a mixture of water, acetonitrile and sodium dodecyl sulfate (65:35:0.4) as the mobile phase at a flow-rate of 1.0 ml min^{-1}, and detection at 210 nm. Fig. 2 shows typical ion-pair reversed-phase HPLC chromatograms obtained from decoctions of Mao-to, Makyo-yokukan-to, Makyo-kanseki-to, Yokuinin-to, Sho-seiryu-to, Keima-kakuhan-to,

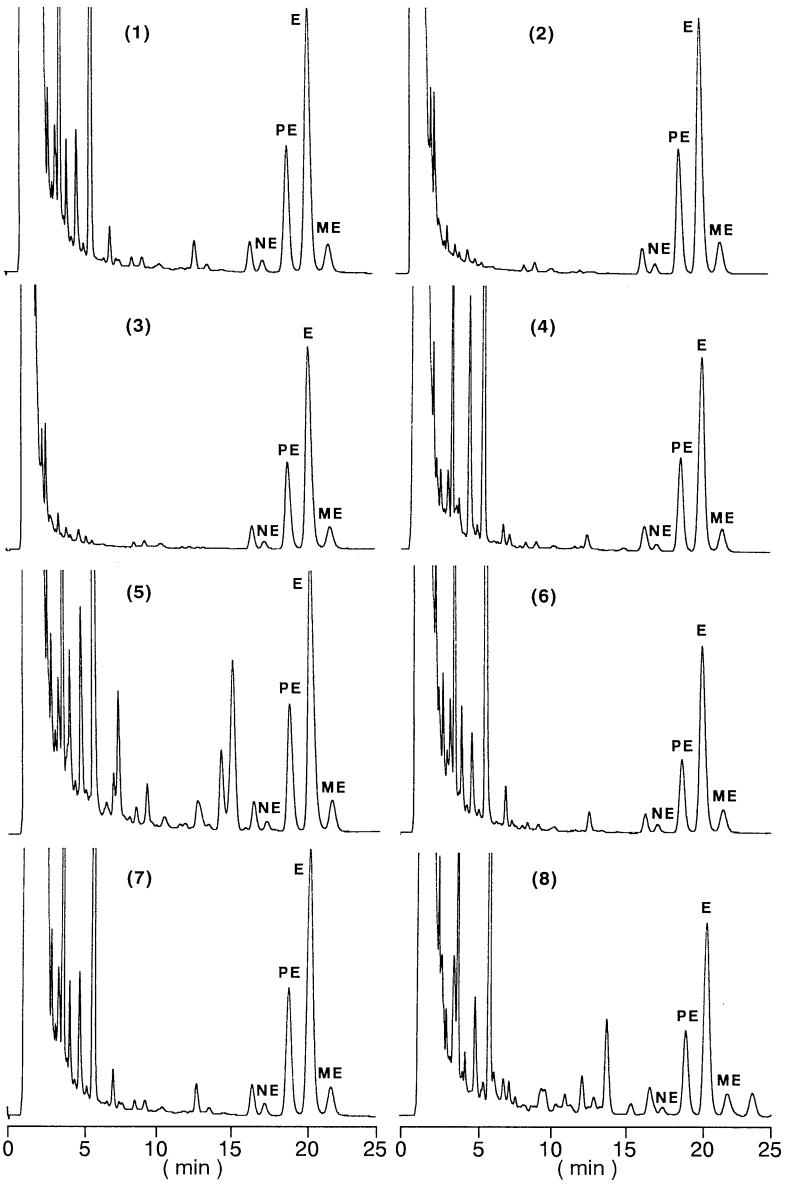

Fig. 2. Chromatograms of Kampo decoctions. Chromatograms: (1) Mao-to; (2) Makyo-kanseki-to (3); Makyo-yokukan-to; (4) Yokuinin-to; (5) Sho-seiryu-to; (6) Keima-kakuhan-to; (7) Kakkon-to; (8) Kakkon-to-ka-senkyu-sin'i. Peaks: NE,norephedrine; PE,pseudoephedrine; E,ephedrine; ME,methylephedrine.

Kakkon-to and Kakkon-to-ka-senkyu-sin'i. Ephedrine, pseudoephedrine, norephedrine and methylephedrine in these decoctions were shown to be sufficiently separated under isocratic conditions without any pre-purification, and were determined within 25 min without any further column purification. The retention times (and capacity factors, k') were 17.44 (norephedrine, $k' = 18.60$), 18.82 (pseudoephedrine, $k' = 20.15$), 20.50 (ephedrine, $k' = 22.03$) and 21.53 min (methylephedrine, $k' = 23.19$). These peaks were identified with standards by inspection of reten-

tion times and UV spectra, and the purity was checked by three-dimensional chromatograms. Conditions described in this method provided distinct elution profiles of ephedrine alkaloids without any interference in the decoctions which were prepared without *Ephedrae Herba* from Mao-to, Makyo-yokukan-to, Makyo-kanseki-to, Yokuinin-to, Sho-seiryu-to, Keima-kakuhan-to, Kakkon-to and Kakkon-to-ka-senkyu-sin'i. The calibration curves of norephedrine, pseudoephedrine, ephedrine and methylephedrine were linear over the ranges of 1.41–560, 1.10–880, 0.93–750 and 0.86–690 μM, respectively. The regression equations were $y = 2192x + 2110$ for norephedrine, $y = 2865x + 3039$ for pseudoephedrine, $y = 2018x + 2059$ for ephedrine and $y = 2402x + 1975$ for methylephedrine with the correlation coefficients of 1.0000, where y is the peak-area and x is the concentrations (μM) of ephedrine alkaloids. These results indicate that the standard curve has a good linearity by linear least-squares regression analysis, and that the method permits the determination of ephedrine alkaloids in Kampo medicines over a relatively wide range of concentrations. The detection limits (signal-to-noise ratio = 3) of norephedrine, pseudoephedrine, ephedrine and methylephedrine were 1.06, 0.37, 0.42 and 0.47 μM, respectively.

Table 2
Within-day and day-to-day relative standard deviations (RSD) for norephedrine, pseudoephedrine, ephedrine and methylephedrine in Mao-to and Sho-seiryu-to

Marker substance	RSD (%)	
	Within-day[a]	Day-to-day[b]
Mao-to		
Norephedrine	1.04	1.84
Pseudoephedrine	0.20	1.05
Ephedrine	0.26	0.99
Methylephedrine	0.33	1.98
Sho-seiryu-to		
Norephedrine	3.46	7.35
Pseudoophedrine	1.73	1.09
Ephedrine	1.71	1.00
Methylephedrine	1.35	0.38

[a] $n = 10$.
[b] $n = 5$.

The within-day and the day-to-day precision of the method for each ephedrine alkaloids were evaluated using decoctions of Mao-to and Sho-seiryu-to, by ten same-day replicate assays and assays on five different days. As Table 2 shows, the within-day and day-to-day relative standard deviations (RSDs) were 0.20–1.73% and 0.38–1.98%, respectively, whereas the RSDs of norephedrine were 3.46 and 7.35%, respectively, in Sho-seiryu-to. The mean recoveries (and RSDs) of standards spiked into decoctions of Mao-to and Sho-seiryu-to were 105.2 (2.86) and 105.1% (2.14) for norephedrine, 101.2 (1.17) and 101.1% (0.50) for pseudoephedrine, 101.1 (0.95) and 101.2% (0.87) for ephedrine, and 100.7 (0.50) and 99.8% (0.43) for methylephedrine, respectively, as shown in Table 3. The mean recoveries of ephedrine alkaloids were within 1.2% of nominal values, showing no significant changes for four spiked concentrations, whereas those of norephedrine were 5.1 and 5.2%. The results indicate this method has both good reproducibility and accuracy.

We applied this procedure to determine ephedrine alkaloids in decoctions of Mao-to, Makyo-yokukan-to, Makyo-kanseki-to, Yokuin-in-to, Sho-seiryu-to, Keima-kakuhan-to, Kakkon-to and Kakkon-to-ka-senkyu-sin'i (Table 4). These Mao-drugs mainly contained ephedrine (more than 70%) and pseudoephedrine (more than 20%), and these ephedrine alkaloids play an important role in the effects. Fig. 3 showed the content ratio (%) of ephedrine alkaloids per *Ephedrae Herba* gram to those in decoctions of Mao-drugs. Ephedrine alkaloids in Makyo-kanseki-to decoction were at higher concentrations than in other decoctions of Mao-drugs. The decoctions were prepared with two to four crude drugs which combined *Gypsum Fibrosum* (GF) (or calcium sulfate: CS), *Cinnamomi Cortex* (CC), *Glycyrrhizae Radix* (GF) and *Armeniacae Semen* (AS) with *Ephedrae Herba* (EH) as shown in Fig. 4. Ephedrine and pseudoephedrine concentrations in decoctions of Mao-to (EH, CC, GR, AS) were low compared with Makyo-kanseki-to (EH, GF, GR, AS), suggesting constituent components prevented the dissolution of ephedrine alkaloids in both the crude drug and Kampo medicine by

Table 3
Recovery of norephedrine, pseudoephedrine, ephedrine and methylephedrine from Mao-to and Sho-seiryu-to

Marker substance	Initial amount (μg/ml)	Added (μg/ml)	Found (μg/ml)	Recovery (%)	Mean ± SD (%)	RSD
Mao-to						
Norephedrine	1.29	3.47	5.12	107.56	105.23 ± 3.01	2.86
		1.74	3.27	107.92		
		0.87	2.24	103.70		
		0.43	1.75	101.74		
Pseudoephedrine	20.27	72.12	94.26	102.02	101.21 ± 1.18	1.17
		36.06	57.61	102.27		
		18.03	38.63	100.86		
		9.02	29.20	99.69		
Ephedrine	68.00	278.35	350.97	101.33	101.09 ± 0.96	0.95
		139.18	211.78	102.22		
		69.59	138.82	100.89		
		34.79	102.69	99.90		
Methylephedrine	7.05	29.73	37.09	100.84	100.72 ± 0.50	0.50
		14.87	22.22	101.37		
		7.43	14.51	100.21		
		3.72	10.82	100.46		
Sho-seiryu-to						
Norephedrine	0.56	3.47	4.22	104.71	105.06 ± 2.25	2.14
		1.74	2.45	106.52		
		0.87	1.53	106.99		
		0.43	1.01	102.02		
Pseudoephedrine	10.95	72.12	83.40	100.40	101.08 ± 0.50	0.50
		36.06	47.66	101.38		
		18.03	29.42	101.52		
		9.02	20.17	101.00		
Ephedrine	39.47	278.35	317.52	99.91	101.21 ± 0.88	0.87
		139.18	181.37	101.52		
		69.59	111.08	101.85		
		34.79	75.41	101.55		
Methylephedrine	3.77	29.73	33.41	99.73	99.84 ± 0.43	0.43
		14.87	18.63	99.95		
		7.43	11.24	100.36		
		3.72	7.44	99.33		

precipitation [12,13] and adsorption [14,15]. We speculate that the inclusion of *Cinnamomi Cortex* instead of *Gypsum Fibrosum* is deeply concerned with either the solubility, the extraction efficiency or the re-absorption to residue of ephedrine and pseudoephedrine in Mao-to decoction, since the concentrations of ephedrine and pseudoephedrine were higher in the decoctions in which *Gypsum Fibrosum* were added. In comparison with absence of *Glycyrrhizae Radix*, large quantities of ephedrine and pseudoephedrine remained in the residue of the decoctions blended with *Glycyrrhizae Radix*. Finally, when *Gypsum Fibrosum* was excluded from Makyo-kanseki-to, the concentrations of ephedrine and pseudoephedrine in the residue remained were similar to the combination decoction (EH, GR, AS). These results show that *Gypsum Fibrosum* may increase the solubility of ephedrine and pseudoephedrine in the decoction before filtering and does not prevent the re-absorption into the residue of these ephedrine alkaloids.

Gypsum Fibrosum consists of mostly calcium sulfate. Ephedrine and pseudoephedrine concentrations were equivalent to those in Makyo-kanseki-to decoction in which calcium sulfate (10

Table 4
Concentrations of norephedrine, pseudoephedrine, ephedrine and methylephedrine in decoctions

Decoction	Norephedrine		Pseudoephedrine		Ephedrine		Methylephedrine	
	Mean ± SD (μg/ml)	RSD (%)	Mean ± SD (μg/ml)	RSD (%)	Mean ± SD (μg/ml)	RSD (%)	Mean ± SD (μg/ml)	RSD (%)
Mao-to	2.69 ± 0.12	4.56	39.94 ± 1.36	3.40	133.42 ± 1.88	1.41	14.00 ± 0.44	3.17
Makyo-yokukan-to	2.57 ± 0.11	4.26	38.85 ± 0.69	1.79	133.11 ± 4.20	3.16	14.26 ± 0.58	4.08
Makyo-kanseki-to	2.95 ± 0.16	5.42	45.30 ± 1.62	3.58	154.14 ± 2.47	1.60	16.59 ± 0.22	1.34
Yokuinin-to	2.35 ± 0.14	5.94	30.62 ± 0.88	2.88	108.44 ± 2.82	2.60	12.89 ± 0.50	3.89
Keima-kakuhan-to	1.28 ± 0.08	6.04	17.45 ± 0.61	3.51	60.52 ± 1.63	2.69	7.03 ± 0.35	4.93
Kakkon-to	2.38 ± 0.12	4.98	32.25 ± 0.80	2.48	114.38 ± 5.26	4.60	13.29 ± 0.59	4.45
Sho-seiryu-to	1.34 ± 0.05	3.92	23.11 ± 1.35	5.82	82.40 ± 2.77	3.37	8.46 ± 0.32	3.78
Kakkon-to-ka-senkyu-sin'i	2.19 ± 0.17	7.84	34.51 ± 1.47	4.26	122.22 ± 4.99	4.08	16.15 ± 0.95	5.86

g) was combined instead of *Gypsum Fibrosum* (Fig. 4), and the addition of an equivalent molar of sodium sulfate, magnesium sulfate, calcium chloride and sodium chloride were similar to those in the decoction with calcium sulfate. These findings suggest that the solubility of ephedrine and pseudoephedrine in decoction may be increased by inorganic salts.

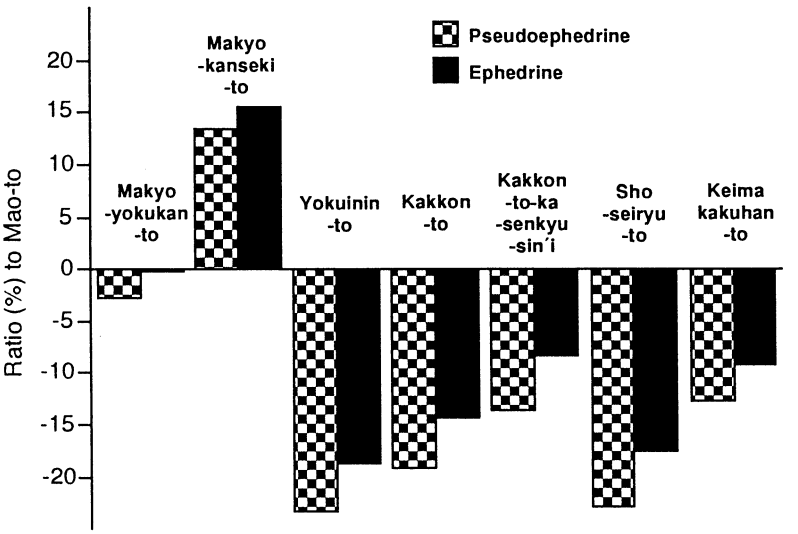

Fig. 3. Percentage of ephedrine alkaloids (per *Ephedrae Herba* gram) in Kampo decoctions for Mao-to.

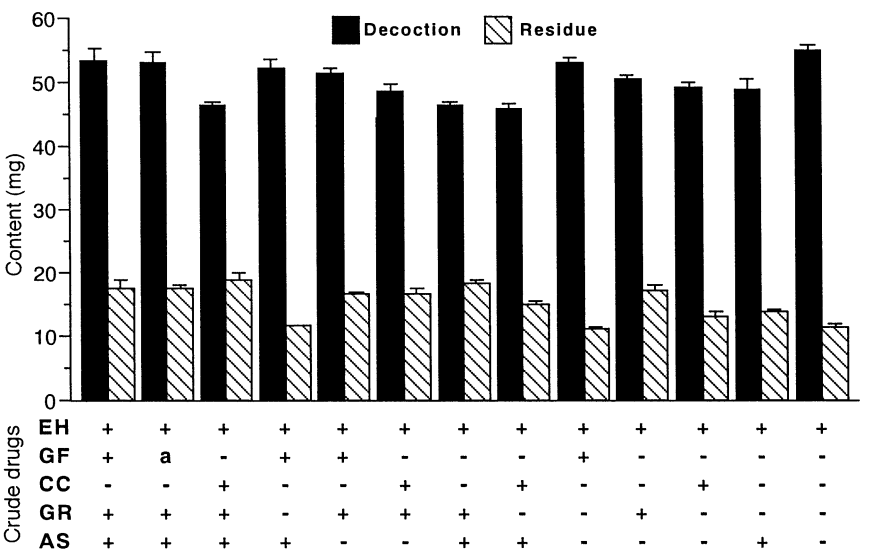

Fig. 4. Contents of ephedrine and pseudoephedrine in decoctions of *Ephedrae Herba* mixed with other crude drugs. a: the symbol means the substitution of GF to CS. Crude drugs: EH, *Ephedrae Herba* (4 g); GF, *Gypsum Fibrosum* (10 g); CS, calcium sulfate (10 g); CC, *Cinnamomi Cortex* (3 g); GR, *Glycyrrhizae Radix* (2 g); AS, *Armeniacae Semen* (4 g).

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