Effect of Mirabilitum in Formularization: Change of Prolyl Endopeptidase Inhibitory Activity and of Constituents Using the Preparation Method of Tokaku-joki-to (桃核承気湯, Persia and Rhubarb Combination)

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To clarify the effect of Mirabilitum in formularization, change of prolyl endopeptidase inhibitory activity and of constituents using the preparation method of a Kampo formula Tokaku-joki-to (桃核承気湯, Persia and Rhubarb combination) was examined by the liquid chromatography–mass spectroscopy (LC-MS) method. Mirabilitum under boiling condition caused qualitative and quantitative change of the constituents through hydrolysis which caused a change of its activity. This was considered to be the main reason the classical Chinese medical book "Shang han lun (傷寒論)" specified that Mirabilitum should be added at a later stage of decoction.

Key words prolyl endopeptidase; Tokaku-joki-to (桃核承気湯); Kampo formula; Mirabilitum; liquid chromatography–mass spectroscopy

In the People's Republic of China and Japan, there are two therapies to treat diseases: Chinese medicine (Kampo medicine in Japan) and modern Western medicine. The former is characterized by the concept of the specific conditions "sho (証, syndrome differentiation)" and the use of a formula "hou-zai (方剤)", corresponding to the "sho" of each patient. The formula is prepared from crude drugs according to the "sho", and some quantitative and/or qualitative change of constituents during preparation of a formula is believed to make the individual treatment feasible. Thus, a chemical examination on the change is necessary for understanding Chinese (Kampo) medicine.

In the course of our search for prolyl endopeptidase (PEP, EC 3.4.21.26) inhibitors from traditional Chinese (Kampo) medicines,[1,2] we found that Tokaku-joki-to (桃核承気湯, Persia and Rhubarb combination) has the PEP inhibitory activity (inhibition rate of 100 μg/ml, 99.6%).[3] Tokaku-joki-to is an oriental blood-quickening and stasis-transforming prescription, which consists of Persicae Semen (桃仁), Rhei Rhizoma (大黄), Cinnamomi Cortex (桂皮), Glycyrrhizae Radix (甘草) and Mirabilitum (芒硝) and is used clinically in the People's Republic of China and Japan for the treatment of amenorrhea, climacteric syndrome, emotional imbalance and so on.[4,5] Our chemical examination of the PEP inhibitory constituents resulted in the identification of twenty-seven constituents including thirteen active ones,[3] which had been reported to be contained in Rhei Rhizoma and/or Cinnamomi Cortex. We thus examined the PEP inhibitory activities of the crude drugs which compose Tokaku-joki-to. In a classical Chinese medical book "Shang han lun (傷寒論)", on the other hand, the decoction method of Tokaku-joki-to has been described as follows (Fig. 1): place Persicae Semen (5.0 g), Rhei Rhizoma (3.0 g), Cinnamomi Cortex (4.0 g) and Glycyrrhizae Radix (1.5 g) in 7 l of water, boil them down into 2.5 l, remove the sediment, add the Mirabilitum and simmer the mixture until dissolved.[4] This description seemed to indicate the qualitative and/or quantitative change of constituents. Thus, we next examined the change using the liquid chromatography–mass spectroscopy (LC-MS) method with the twenty-seven compounds (**1**—**27**, Fig. 2) isolated from Tokaku-joki-to as standards.

Experimental

Preparation of Extracts of Crude Drugs and Formulas Rhei Rhizoma (*Rheum palmatum* LINNE, Sichuan Province in China), Cinnamomi Cortex (*Cinnamomum cassia* BLUME, Guangdong Province in China), Persicae Semen (*Prunus persica* BATSCH, Shanxi Province in China), Glycyrrhizae Radix (*Glycyrrhiza uralensis* FISHER, Hebei Province in China) and Natrium Sulfuricum (Na_2SO_4) were supplied by Kotaro Kampo Pharmaceutical Co., Ltd., Osaka, Japan, while Magnesium Sulfuricum ($MgSO_4$) was purchased from Nacalai Tesque Co. (Kyoto, Japan).

Each crude drug was extracted 3 times with 1 l of boiling water for 1 h. The extracts were combined, concentrated *in vacuo* and then freeze-dried. The yield of each extract from *R. palmatum*, *C. cassia*, *G. uralensis* and *P. persica* was 13.4, 4.5, 15.5 and 11.2%, respectively (Table 1).

Formula **A0** was prepared by the method described in a classical Chinese medical book "Shang han lun (傷寒論)" (Fig. 1)[4] with a yield of 21.6%. Formula **A1** was prepared by decocting the mixture of Rhei Rhizoma, Cinnamomi Cortex, Persicae Semen and Glycyrrhizae Radix with distilled water (700 ml) for 1 h, followed by filtration of the mixture, concentration of the filtrate to 250 ml and lyophilization; yield, 16.0%. Formulas **A2** and **A3** were prepared by decocting the crude drugs with Na_2SO_4 (**A2**) or $MgSO_4$ (**A3**) with the same procedure as formula **A1** with a yield of 20.0% (**A2**) or 17.4% (**A3**). Formula **A4** was prepared by boiling formula **A1** with Na_2SO_4 for 30 min, followed by lyophilization.

Mixtures **B1**—**B4** were prepared by grounding the extracts of the crude drugs corresponding to the above formulas **A1**—**A4**; ratio of each extract (Table 1) was calculated based on the yield from each crude drug. Extracts **C1** and **C2** were prepared by boiling the extracts from Rhei Rhizoma and Cinnamomi Cortex with Na_2SO_4 for 1 h.

PEP Inhibitory Activity PEP (*Flavobacterium meningosepticum* origin) was purchased from Seikagaku Corporation (Tokyo, Japan) and a substrate, *Z*-Gly-Pro-*p*NA, from Bachem Fine Chemical Co. (Switzerland). PEP inhibitory activities were measured by the method of Yoshimoto *et al.*[6] as described in our previous papers.[1—3]

LC-MS Analysis Reference standards [cinnamic acid (**1**), protocatechuic acid (**2**), gallic acid (**3**), torachrysone 8-*O*-β-D-glucoside (**4**), emodin (**5**), emodin 8-*O*-β-D-glucopyranoside (**6**), *trans*-3,5,4′-trihydroxystilbene 4′-*O*-β-D-glucopyranoside (**7**), *trans*-3,5,4′-trihydroxystilbene 4′-*O*-β-D-(2-*O*-galloyl)glucopyranoside (**8**), *trans*-3,5,4′-trihydroxystilbene 4′-*O*-β-D-(6-*O*-galloyl)glucopyranoside (**9**), *cis*-3,5,4′-trihydroxystilbene 4′-*O*-β-D-(6-*O*-galloyl)glucopyranoside (**10**), 4-(4-hydroxyphenyl)-2-butanone 4′-*O*-β-D-

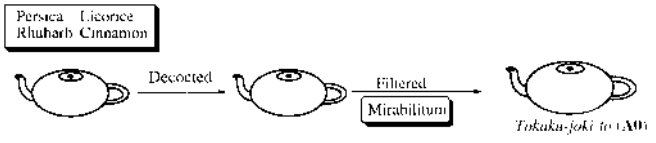

Fig. 1. Decoction Procedure According to the Classical Chinese Medical Book "Shang han lun"

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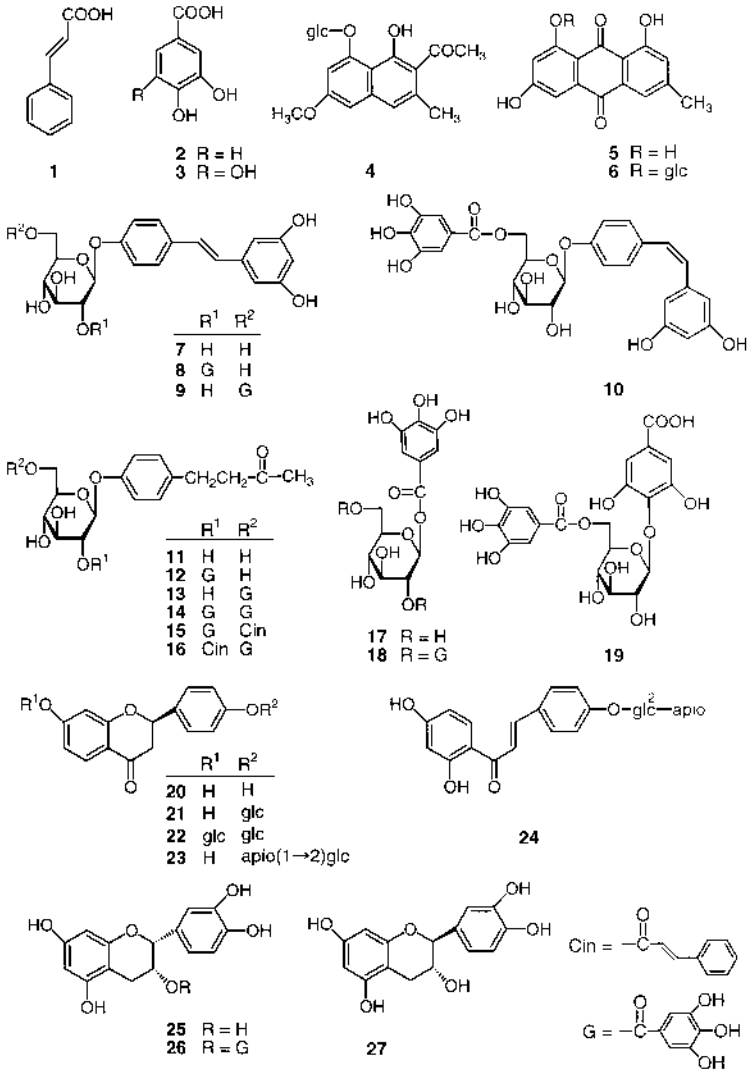

Fig. 2. Structures of Isolated Compounds from a Commercial Kampo Formula Tokaku-joki-to

Glc: β-D-glucopyranosyl, apio: β-D-apiofuranosyl.

Table 1. Preparation of Extracts of Crude Drugs and Formulas

Name	Constituent	Extract time (min)	Weight (g)
Persicae Semen	100 g	60	11.2
Cinnamomi Cortex	100 g	60	4.5
Rhei Rhizoma	100 g	60	13.4
Glycyrrhizae Radix	100 g	60	15.5
A0	Persicae Semen (34.7 g), Cinnamomi Cortex (27.8 g), Rhei Rhizoma (20.8 g), Glycyrrhizae Radix (10.4 g), Na_2SO_4 (6.3 g)	60	21.6
A1	Persicae Semen (37.0 g), Cinnamomi Cortex (29.6 g), Rhei Rhizoma (22.2 g), Glycyrrhizae Radix (11.1 g)	60	16.0
A2	Persicae Semen (34.7 g), Cinnamomi Cortex (27.8 g), Rhei Rhizoma (20.8 g), Glycyrrhizae Radix (10.4 g), Na_2SO_4 (6.3 g)	60	20.0
A3	Persicae Semen (34.7 g), Cinnamomi Cortex (27.8 g), Rhei Rhizoma (20.8 g), Glycyrrhizae Radix (10.4 g), MgSO4 (6.3 g)	60	17.4
A4	**A1** (2.0 g)+Na_2SO_4 (7.8 mg)	30	2.01
B1[a)]	Persicae Semen (0.56 g), Cinnamomi Cortex (0.14 g), Rhei Rhizoma (0.54 g), Glycyrrhizae Radix (0.23 g)	60	1.46
B2[a)]	Persicae Semen (0.56 g), Cinnamomi Cortex (0.14 g), Rhei Rhizoma (0.54 g), Glycyrrhizae Radix (0.23 g), Na_2SO_4 (0.9 g)	60	2.36
B3[a)]	Persicae Semen (0.56 g), Cinnamomi Cortex (0.14 g), Rhei Rhizoma (0.54 g), Glycyrrhizae Radix (0.23 g), $MgSO_4$ (0.9 g)	60	2.36
B4	**B1** (1.0 g)+Na_2SO_4 (0.62 g)	30	1.62
C1	Rhei Rhizoma (3.0 g)+Na_2SO_4 (0.9 g)	60	2.44
C2	Cinnamomi Cortex (4.0 g)+Na_2SO_4 (0.9 g)	60	0.48

a) The amount of each extract in mixtures **B1**—**B3** was calculated from the extraction yield (%) and ratio of the crude drug in formula **A1**—**A3**, respectively.

glucopyranoside (**11**), isolindleyin (**12**), lindleyin (**13**), 4-(4-hydroxyphenyl)-2-butanone 4′-*O*-*β*-D-(2,6-di-*O*-galloyl)glucopyranoside (**14**), 4-(4′-hydroxyphenyl)-2-butanone 4′-*O*-*β*-D-(2-*O*-galloyl-6-*O*-cinnamoyl)glucopyranoside (**15**), 4-(4′-hydroxyphenyl)-2-butanone 4′-*O*-*β*-D-(2-*O*-cinnamoyl-6-*O*-galloyl)glucopyranoside (**16**), 1-*O*-galloylglucose (**17**), 1,2,6-tri-*O*-galloylglucose (**18**), gallic acid 4-*O*-*β*-D-(6-*O*-galloyl)glucopyranoside (**19**), liquiritigenin (**20**), liquiritigenin 4′-*O*-*β*-D-glucopyranoside (**21**), liquiritigenin 7,4′-diglucoside (**22**), liquiritigenin 4′-*O*-*β*-D-apiofuranosyl-(1→2)-*β*-D-glucopyranoside (**23**), licuroside (**24**), (−)-epicatechin (**25**), (−)-epicatechin 3-*O*-gallate (**26**), (+)-catechin (**27**); Fig. 1] were isolated from EtOAc-soluble and BuOH-soluble fractions of a commercial Tokaku-joki-to by a combination of column chromatography and preparative thin layer chromatography (TLC), as described.[3)]

Each sample was dissolved in HPLC-grade H_2O (10 mg/ml) and filtered using a HPLC-Disk 13 filter (Kanto Chemical Co. Inc, Japan), and 10 μl of the filtrate was directly subjected to LC-MS analysis. The analysis was performed on a JEOL JMS-700T mass spectrometer (Tokyo), equipped with a HP1100 liquid chromatograph (Hewlett-Packard, Germany), with an atmospheric pressure chemical ionization (APCI) mode: temperature, 400 °C; ion injection time, 0.1 s; column, Supelco discovery C_{18} (4.6×250 mm; U.S.A.); mobile phase, 10 mM AcOH–CH_3CN (linear gradient, 95 : 5→35 : 65 for 60 min); flow rate, 0.5 ml/min; UV, 220 nm.

Results and Discussion

In the previous paper,[3)] we determined the PEP inhibitory constituents in a commercial Tokaku-joki-to to be **2**, **7**—**10**, **14**—**16**, **18**, **19** and **24**—**26**, which had been reported as constituents of Rhei Rhizoma (**1**—**19**, **25**—**27**) and Cinnamomi Cortex (**1**, **25**—**27**). We thus examined the PEP inhibitory activity of each crude drug in order to learn which one contributes to the activity of Tokaku-joki-to. As can be seen in Table 2, Rhei Rhizoma and Cinnamomi Cortex showed strong inhibitory activity (IC_{50}, 0.73 and 2.72 μg/ml, respectively), while Persicae Semen, Glycyrrhizae Radix and Natrium Sulfuricum showed no activity. The inhibitory activity of the former two were stronger than that of the formula Tokaku-joki-to (**A2**; IC_{50}, 12.9 μg/ml), suggesting that combination of the crude drugs could affect the PEP inhibitory activity. In addition, Magnesium Sulfuricum ($MgSO_4$), which was at one time used as Mirabilitum in Japan,[7)] showed the same weak PEP inhibitory activity as Natrium Sulfuricum (Na_2SO_4) which is used these days.

The commercial Tokaku-joki-to, which we used in the previous examination,[3)] was prepared by the same method as formula **A2**. However, as described above, the method differed from the original one described in the classical Chinese medical book “Shang han lun (傷寒論)” in the time of adding Mirabilitum: in formula **A2** Mirabilitum was mixed from the first and boiled; in formula **A0** it was mixed after the decoction of the other four crude drugs. Thus, we focused on the effect of Mirabilitum on the change of PEP inhibitory activity and the constituents.

Formula **A1** without Mirabilitum showed stronger inhibitory activity than the original formula **A0** with Mirabilitum (Na_2SO_4), and formulas **A2** and **A3** (containing Na_2SO_4 and $MgSO_4$, respectively, as Mirabilitum) showed significant decrease of the inhibitory activity (Table 2). These results indicated that the presence of Mirabilitum (Na_2SO_4 or $MgSO_4$) decreased the PEP inhibitory activity, which was also observed in the case of mixtures of extracts (**B1**—**B3**). In these experiments, the PEP inhibitory activities of formulas **A2** and **A3** and of mixtures **B2** and **B3** were almost the same, indicating that there should be no difference between Natrium Sulfuricum (Na_2SO_4) and Magnesium Sulfuricum ($MgSO_4$). Thus, we used Natrium Sulfuricum (Na_2SO_4) as Mirabilitum in the subsequent experiments.

Table 2. PEP Inhibitory Activity of Formulas and Crude Drugs

Name	Conc. (μg/ml)	Inhibition (%)	IC_{50} (μg/ml)
Rhei Rhizoma	100	99.3±0.5	0.73
	10	98.1±1.1	
	1	57.0±17	
	0.1	11.8±5.8	
Cinnamomi Cortex	100	98.2±0.8	2.72
	10	83.0±3.9	
	1	24.6±5.4	
Persicae Semen	100	23.9±6.3	
Glycyrrhizae Radix	100	5.0±4.0	
Natrium Sulfuricum	100	3.1±1.9	
Magnesium Sulfuricum	100	2.2±2.9	
A0	100	96.8±2.4	2.40
	10	88.6±4.2	
	1	26.3±7.1	
A1	100	99.6±0.4	1.86
	10	98.5±0.3	
	1	32.1±5.1	
A2	100	99.5±0.4	12.9
	10	43.9±2.9	
	1	19.4±2.2	
A3	100	97.9±1.3	15.3
	10	39.4±6.5	
	1	12.6±6.9	
A4	100	98.1±2.7	6.52
	10	58.1±7.9	
	1	16.5±6.4	
B1	100	85.6±7.6	15.0
	10	45.0±1.7	
	1	20.8±1.8	
B2	100	83.5±0.9	25.9
	10	26.4±4.3	
	1	11.9±2.9	
B3	100	86.3±1.9	22.8
	10	29.1±3.0	
	1	8.5±4.6	
B4	100	64.0±1.9	49.0
	10	18.5±1.6	
	1	9.0±3.5	
C1	100	100±0.0	2.19
	10	99.8±0.2	
	1	24.4±2.6	
C2	100	98.9±1.1	13.0
	10	43.8±4.8	
	1	17.0±5.9	

The values are the means±S.D. of triplicate experiments.

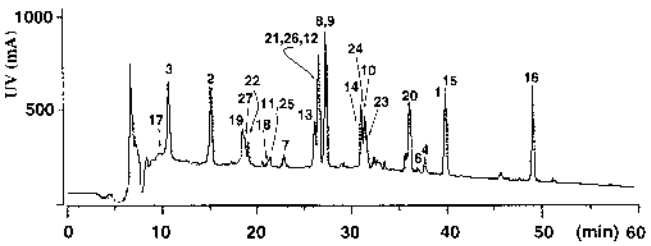

Fig. 3. HPLC Profile of a Mixture of Standard Compounds **1**—**27** Except for **5**

Then, we examined the change of the constituents with HPLC using both UV and APCI-MS detections. The HPLC profile of a mixture of standard compounds with UV detection showed clear separation of almost all of them (Fig. 3). Compounds **11** and **25**, compounds **12**, **21** and **26** and compounds **1** and **15**, which could not be separated by UV detec-

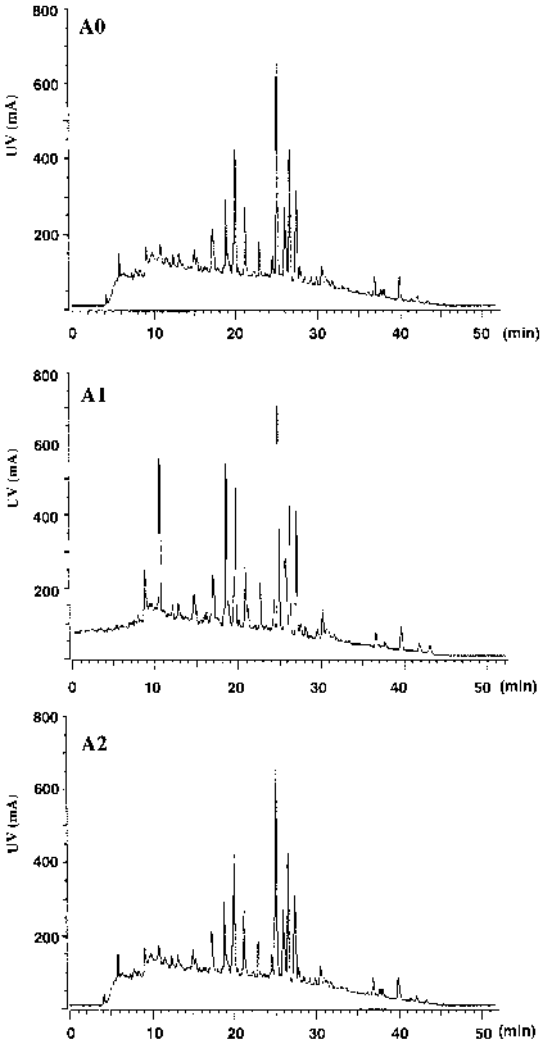

Fig. 4. HPLC Profiles of the Water Solution of Formulas **A0**—**A2**

Formula **A0** consisted of Persicae Semen, Cinnamomi Cortex, Glycyrrhizae Radix, Rhei Rhizoma, Mirabilitum (Na_2SO_4) and was prepared by the method described in the classical Chinese medical book "Shang han lun". Formula **A1** was prepared by decocting the crude drugs other than Mirabilitum (Na_2SO_4). Formula **A2** was prepared by decocting the crude drugs including Mirabilitum (Na_2SO_4).

tion, were also separated in the mass chromatograms of the quasimolecular ions due to their different molecular weights. The HPLC profiles of formulas **A0**—**A2** with UV detection clearly showed the decrease of the amount of constituents in the order **A1**>**A0**>**A2** (Fig. 4). In the mass chromatograms of the quasimolecular ions for compounds **1**—**27** in formula **A1** (without Na_2SO_4), compounds **1**, **7**, **11**—**13**, **18**, **21**—**23**, **25** and **27** were detected, but those of the formula **A2** (with Na_2SO_4) indicated their quantity was decreased; compounds **13**, **22**, **25** and **27** could not be detected (Table 3). Similar reduction of the constituents was also observed in the original formula **A0** when Na_2SO_4 was added later. Thus, Natrium Sulfuricum (Na_2SO_4) should bring a quantitative change of the constituents, which would induce the weakening of the PEP inhibitory activity.

The difference between formulas **A1** and **A2**/**A3** was larger than that between mixtures **B1** and **B2**/**B3**, and formula **A1** showed slightly stronger activity than the original formula **A0**. The large difference between these formulas and mixtures was the heating period with Mirabilitum: formula

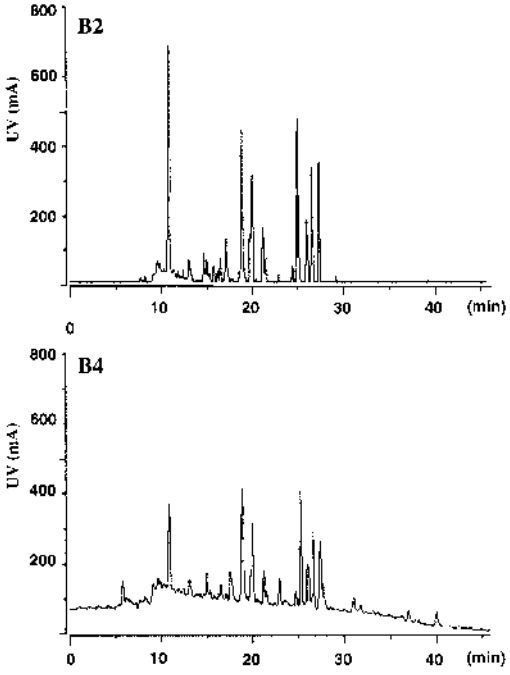

Fig. 5. HPLC Profiles of the Water Solutions of Mixtures **B2** and **B4**

Mixtures **B2** and **B4** were prepared by grounding the extracts of the crude drugs corresponding to formulas **A2** and **A4**, respectively.

A1 and mixture **B1** were not heated with Mirabilitum; original formula **A0** and mixtures **B2** and **B3** were slightly heated to dissolve Mirabilitum; and formulas **A2** and **A3** were decocted with Mirabilitum. Thus, the heating process with Mirabilitum seemed to contribute to the reduction in PEP inhibitory activity. In order to verify this, we boiled formula **A1** with Na_2SO_4 for 30 min to get formula **A4**, while mixture **B2** containing Na_2SO_4 was boiled for 30 min to give mixture **B4**. The heating with Na_2SO_4 decreased the PEP inhibitory activity (Table 2), and the HPLC profile with UV detection showed the decrease in the amounts of the constituents (Fig. 5). In the mass chromatograms of the quasimolecular ions of compounds **1**—**27** of mixture **B2**, compounds **3**, **7**, **11**, **12**, **20** and **27** were detected (Table 3). The mass chromatograms of mixture **B4** also showed the decrease of compounds **3**, **11**, **12**, **20** and **27**, but interestingly, the amounts of compounds **7** and **21** were increased and compound **1** was newly detected. From these data, together with consideration of their structures, gallates and/or glycosides such as **8**, **9**, **22** and **23** were hydrolyzed to give compounds **7** and **21**, while **1** may be derived from cinnamic acid esters such as **15** and **16**, *i.e.*, Mirabilitum (Na_2SO_4) in the formulas, under the heating condition, brought about a qualitative and quantitative change in the constituents.

This was further confirmed by examining the effect of Mirabilitum (Na_2SO_4) under boiling condition on the extracts from Rhei Rhizoma and Cinnamomi Cortex. The PEP inhibitory activity of the extracts **C1** and **C2**, prepared by boiling the extracts from Rhei Rhizoma and Cinnamomi Cortex with Na_2SO_4, was significantly decreased (Table 2). In addition, the HPLC chromatograms of the extracts **C1** and **C2** with UV detection showed the quantitative and qualitative decrease of the constituents compared with those of Rhei Rhizoma and Cinnamomi Cortex, respectively (Fig. 6). In accordance with this, the mass chromatograms of the quasimol-

Table 3. Mass Number of Quasimolecular Ion, Retention Time (t_R) and Relative Intensity of the Quasimolecular Ion of the Compounds **1**—**27**

Compound	*m/z*	t_R (min)	**A0**	**A1**	**A2**	**B2**	**B4**	Rhei Rhizoma	**C1**	Cinnamomi Cortex	**C2**
1	147	39.9	2590	726	282	—	2630	—	—	119000	7360
2	153	15.3	—	—	—	—	—	454	—	—	—
3	169	10.8	—	—	—	605	—	17100	48000	—	—
4	411	37.4	—	—	—	—	—	17700	2050	—	—
6	431	36.9	—	—	—	—	—	1240	—	—	—
7	389	22.9	349	22300	1560	1050	4860	10300	6450	—	—
8, **9**	541	27.5	—	—	—	—	—	41300	8030	—	—
11	325	21.3	7870	60100	6560	14000	—	7570	3160	—	—
12	477	27.0	—	21000	445	2950	—	581000	340000	—	—
13	477	26.2	—	500	—	—	—	166000	16200	—	—
14	629	30.8	—	—	—	—	—	674	—	—	—
17	331	9.3	—	—	—	—	—	35000	25700	—	—
18	635	20.9	1000	4173	324	—	—	298	—	—	—
19	483	18.5	—	—	—	—	—	1280	435	—	—
20	255	36.3	—	—	—	1460	—	—	—	—	—
21	417	26.7	10000	87100	4040	14200	31400	—	—	—	—
22	579	19.3	—	1040	—	—	—	—	—	—	—
23	537	32.0	656	5370	3650	—	—	—	—	—	—
25	289	21.7	—	290	—	—	—	65600	9680	—	—
26	441	26.6	—	—	—	—	—	41700	4120	2200	—
27	289	18.8	—	290	—	867	—	459300	407000	—	—

Identification of compounds was based on comparison of their retention time and molecular weight compounds **5** (*m/z* 269; t_R 62.0), **10** (*m/z* 541; t_R 31.6), **15** (*m/z* 607; t_R 40.1), **16** (*m/z* 607; t_R 49.1) and **24** (*m/z* 537; t_R 30.9) could not be detected due to their small amounts.

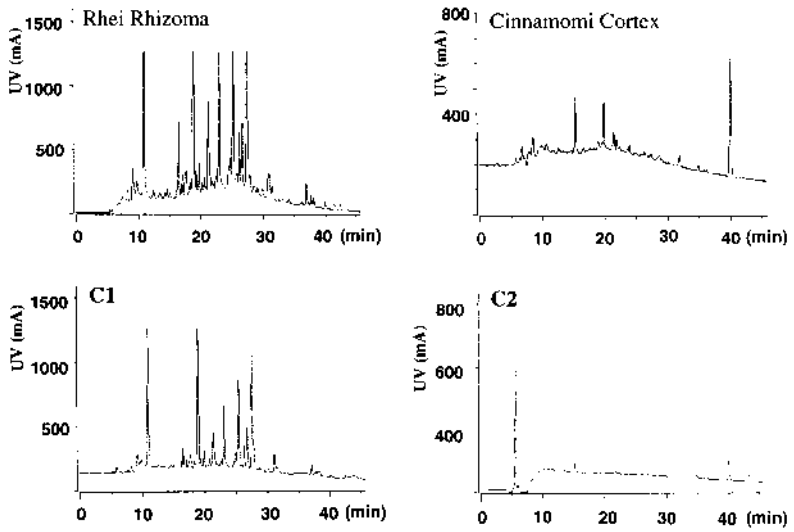

Fig. 6. HPLC Profiles of the Water Solutions of Extracts from Rhei Rhizoma and Cinnamomi Cortex and of Extracts **C1** and **C2**

Extracts **C1** and **C2** were prepared by boiling the extracts from Rhei Rhizoma and Cinnamomi Cortex with Na_2SO_4, respectively.

ecular ions of compounds **2**, **4**, **6**—**9**, **11**—**14**, **17**—**19** and **25**—**27** indicated the decrease of their quantity in the extract **C1**, but that of gallic acid (**3**) increased in extract **C1** (Table 3). A similar tendency was also observed in the mass chromatograms of Cinnamomi Cortex and the extract **C2**. From these data, it was concluded that Mirabilitum (Na_2SO_4) under boiling condition caused the quantitative and qualitative change of constituents through hydrolysis to decrease the PEP inhibitory activity.

Conclusion

In this paper, we have reported that Mirabilitum under boiling condition changed the constituents qualitatively and quantitatively through hydrolysis and caused a change in their activity. Thus, to avoid hydrolysis by Mirabilitum, the classical Chinese medical book “Shang han lun” specified that Mirabilitum should be added at a later stage of decoction. Interestingly, the same specification was indicated on other formulas containing Mirabilitum, such as Choi-joki-to (調胃承気湯, Rhubarb and Mirabilitum Combination), Daiobotanpi-to (大黄牡丹皮湯, Rhubarb and Moutan Combination) and Saiko-ka-bosho-to (柴胡加芒硝湯, Bupleurum and Mirabilitum Combination),[8] suggesting that the same phenomenon occurred.

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