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Short communication

Quantitative determination of potent α-glucosidase inhibitors, salacinol and kotalanol, in *Salacia* species using liquid chromatography–mass spectrometry

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ABSTRACT

A practical HPLC–MS method for the quantitative determination of salacinol (**1**) and kotalanol (**2**), potent α-glucosidase inhibitors from *Salacia* species (Hippocrateaceae) as a specific remedy for diabetes in Ayurvedic system, was developed. The optimum conditions of separation and detection of these two constituents were achieved on a Asahipak NH2P-50 column (5 μm particle size, 2.0 mm i.d. × 150 mm) with a CH_3CN–H_2O mobile phase, associated with MS using electrospray ionization source. The overall recoveries of **1** (85.8–112.6%) and **2** (99.7–106.1%), and relative standard deviation values of intra- and inter-day precision were lower than 6.8 and 8.5%, respectively. The detection (S/N = 3) and quantitation limits (S/N = 10) were established to be 0.015 and 0.050 ng for **1**, and 0.030 and 0.10 ng for **2**, respectively. The correlation coefficients of all the calibration curves showed good linearity within test ranges. The extraction process was also optimized as 2 h immersion in water under reflux. The method was applied to evaluate extracts of three kinds of *Salacia* species, i.e. *S. reticulata*, *S. oblonga*, and *S. chinensis*, and those of four different parts, i.e. roots, stems, leaves and fruits of the same material, revealing that the extract from the roots of *S. reticulata* had the highest contents of these compounds. The results indicated that the assay was reproducible and precise and could be readily utilized for the evaluation of *Salacia* species.
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1. Introduction

Genus *Salacia* (Hippocrateaceae) are woody climbing plants and widely distributed in India, Sri Lanka, China and Southeast Asian countries such as Thailand, Indonesia, and also in a torrid zone area such as Brazil [1,2]. Among them, the roots and the stems of *Salacia* (*S.*) *reticulata* and *S. oblonga* have extensively been used for treatment of rheumatism, gonorrhea, skin diseases, and particularly as a specific remedy for diabetes of early stage in Ayurvedic system, the Indian traditional folk medicine [2]. In Thailand, stems of *S. chinensis* have been used as a laxative as well as an antidiabetic and also for relaxation of myalgia [1]. In the course of our studies on bioactive constituents from natural medicines and medicinal foodstuffs, the authors isolated two novel sulfoniums termed salacinol (**1**) [3,4] and kotalanol (**2**) [5] with substantial hypoglycemic effects on sucrose- and maltose-loaded rats [3–6] from the roots and stems of *Salacia* plants. The mechanism of action was proved to be α-glucosidase inhibition, and the inhibitory activities of **1** [IC_{50} values (μg/mL): 0.42 for sucrase and 2.0 for maltase] and **2** (0.18 and 0.86) were revealed to be as high as those of acarbose (1.0 and 1.1), which is widely used clinically these days [3–5]. Besides, the common structure was quite unique, bearing thiosugar sulfonium sulfate inner salt comprised of 1-deoxy-4-thio-D-arabinofuranosyl cation and 1-deoxy-aldosyl-3-sulfate anion as shown in Fig. 1. Because of their intriguing structure and high α-glucosidase inhibitory activities, intensive structure–activity relationship (SAR) studies have also been reported [1,7–10].

On the other hand, owing to increasing interests in *Salacia* as a possible nutraceutical product for diabetic patients, there is a strong demand for efficient quality control measurement to ensure the authenticity and content of the active constituents in these products, and to verify the labeled claims. In this paper, we propose a simple, rapid, and precise analytical method for quantitative determination of these two sulfoniums (**1** and **2**) using a one-step sample preparation procedure. Since **1** and **2** lack the characteristic chromophore necessary for UV detection, liquid chromatography–mass spectrometry (LCMS) technique was employed.

2. Experimental

2.1. Chemicals and solvents

Acetonitrile and distilled water for LCMS were purchased from Nacalai Tesque Inc., Japan. All other chemicals including authen-

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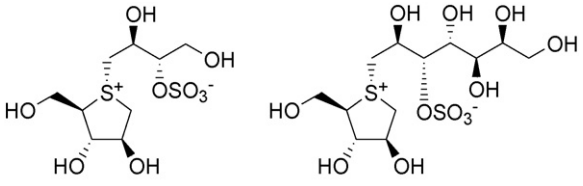

Fig. 1. Structures of salacinol (**1**) and kotalanol (**2**).

tic dulcitol (**3**) were reagent grade, and were purchased from Wako Pure Chemical Industries, Ltd., Japan or Nacalai Tesque Inc., Japan. Diaion HP-20, Chromatorex NH (100–200 mesh), and Duolite A368S were purchased from Nippon Rensui Co., Japan, Fuji Silysia Chemical, Ltd., Japan, and Sumitomo Chemical Co., Ltd., Japan, respectively.

2.2. Plant material

The roots of *S. reticulata* were collected in Sri Lanka at 2007. The roots of *S. oblonga* were collected in India at 2006. A piece of whole plant of *S. chinensis* collected in Thailand at 2008 was sorted into the roots, the stems, and the leaves, and subjected to extraction. The fruits of *S. chinensis* were collected in Thailand at 2008. These plant materials were identified by one of the authors (M.Y.), and voucher specimens of them are on file in our laboratory.

2.3. Isolation of standards

Practical protocol for isolation of **1** and **2** was newly developed in the present study by modifying the previously reported method [3–5] as presented in the supplemental data.

2.4. Standard and sample solutions

2.4.1. Standard solution

Accurately weighed 2.00 mg of **1** or **2** was introduced into a 20 mL volumetric flask and made up to the volume with water, the solution being used as a stock standard solution (100 μg/mL). Aliquots of 0.1, 0.2, 0.3, 0.5, and 1.0 mL of the stock standard solution were transferred into a 20 mL volumetric flask and made up to the volume with 50% MeOH, the solutions being used as working solutions (0.5, 1.0, 1.5, 2.5, and 5.0 μg/mL, respectively) for constructing calibration curves. For the calibration an aliquot of 1 μL of each solution was injected into the LCMS system.

2.4.2. Sample solution

Prior to the analyses, extraction conditions to optimize the extracts' quality in association with contents of the active constituents (**1** and **2**) were examined. Thus, the extraction efficacies were compared by three solvent systems (water, 50% methanol, or methanol) under two different conditions (reflux for 120 min or sonication for 30 min, each twice). As the result, "reflux in water" afforded the highest extraction yield (8.5% from dried roots), and the highest contents of **1** and **2** [relative value (%) against content in water under reflux: 50% MeOH under reflux (**1**: 103%, **2**: 96%); MeOH under reflux (**1**: 86%, **2**: 60%); water under sonication (**1**: 99%, **2**: 96%); 50% MeOH under sonication (**1**: 97%, **2**: 95%); and MeOH under sonication (**1**: 57%, **2**: 33%)]. Therefore, all the analytical samples were prepared by employing the method "reflux in water for 120 min". Thus, an accurately weighed pulverized sample (*ca.* 2 g, conversion with loss on drying) was extracted with 20 mL of water heated under reflux for 120 min. The extraction was repeated twice. After centrifugation of the extracts at 3000 rpm for 5 min, the supernatants were combined and diluted to 100 mL with the extraction solvent. The solution was filtered through a syringe filter (0.45 μm), and an aliquot of 1 μL was subjected to LCMS analysis.

2.5. LCMS instruments and conditions

A series LC-20A Prominence HPLC system (Shimadzu, Kyoto, Japan) was equipped with a binary pump, a degasser, an autosampler, a thermostated column compartment and a control module connected with a LCMS-2010EV mass spectrometer (Shimadzu, Kyoto, Japan) equipped with an electrospray ionization (ESI) interface. The chromatographic separation was performed on an Asahipak NH2P-50 column (5 μm particle size, 2.0 mm i.d. × 150 mm, Showa Denko K.K., Japan) operated at 40 °C. The mobile phase was consisted of acetonitrile and water (78:22, v/v), and was delivered at a flow rate of 0.2 mL/min. The injection volume was 1 μL. The mass spectrometer was operated at negative mode with selected ion monitoring (SIM). The optimal operating parameters of ESI for the maximum signal intensity of the molecular ions were obtained by direct infusion of the standard solutions of **1** and **2**, and were as follows; neburizing gas flow: 1.5 L/min, drying gas pressure: 0.15 MPa, CDL temperature: 250 °C, block heater temperature: 200 °C, CDL voltage: Constant-mode (−25 V), Q-array DS & RF voltage: Scan-mode. Under SIM mode, deprotonated molecular ions ($[M-H]^-$) for each compound were observed at following retention times (**1** *m/z* 333, t_R 8.0 min; **2** *m/z* 423, t_R 14.8 min; and **3** *m/z* 181, t_R 5.8 min).

2.6. Calibration and validation

The standard curve was prepared over a concentration range of 0.5–5.0 μg/mL with five different concentration levels. Standard curves were made on each analysis day. Linearity for each compound was plotted using linear regression of the peak area versus concentration. The coefficient of correlation (R^2) was used to judge the linearity. The detection limits and quantitation limits for each analyte were determined by the signal-to-noise (S/N) ratio for each compound by analyzing a series of diluted standard solutions until the S/N ratios were about 3 and 10, respectively, based on a 1 μL injection. Precision and accuracy of the analytical method were tested using a homogeneous extract of the stems of *S. chinensis*. The intra- and inter-day precisions were determined by estimating the corresponding responses five times on the same day and on five different days, respectively. The recovery rates were determined by adding analytes of three different concentrations (1, 2, and 3 μg/mL) to a sample solution.

2.7. Bioassay method: rat small intestinal α-glucosidase inhibitory activity

Inhibitory effect on rat small intestinal α-glucosidase was assayed by the method described in our previous reports [3–6] with slightly modification. Practical protocol was presented in the supplemental data.

3. Results and discussion

3.1. Column selection

Due to their highly hydrophilic properties, **1** and **2** did not retain in most columns used, and some slight peak tailings were observed. Finally, several amino columns such as Cosmosil Sugar-D (Nacalai Tesque Inc., Japan), CAPCELL Pak NH2 (Shiseido Co., Ltd., Japan), and Asahipak NH2P-50 (Showa Denko K.K., Japan) were found applicable to the analysis, showing appropriated retention times with respect to **1** and **2** by using CH_3CN–H_2O as a mobile phase. Among them, Asahipak NH2P-50 and CAPCELL Pak NH2 gave excellent peak

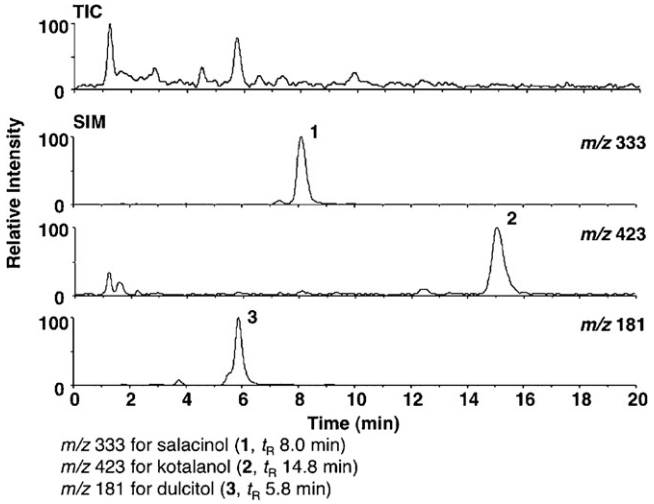

Fig. 2. Typical TIC and SIM chromatograms (negative-ESI–MS) of a water extract from roots of *S. reticulata*.

shape and good separation of **1** and **2** from other concomitants in the extract. By the Cosmosil Sugar-D and CH_3CN–H_2O system, compound **1** was hardly separable from dulcitol (**3**), which is a major sugar alcohol component in *S. reticulata* [4]. Thus, Asahipak NH2P-50 (2.0 mm i.d. × 150 mm) with CH_3CN–H_2O (78:22, v/v) as the eluent was employed in the present study.

3.2. MS condition

The first step in the development of the LCMS procedure was to determine the type of ionization. Standard solutions were tested on electrospray ionization (ESI) under both positive and negative ion modes. As the result, quasimolecular ion ($[M-H]^-$) peaks (*m*/*z* 333 for **1** and *m*/*z* 423 for **2**, respectively) were clearly observed under the negative ion mode, while under the positive mode, the quasimolecular ion peak for **2** (*m*/*z* 425 $[M+H]^+$) could not be detected (data not shown), although one for **1** (*m*/*z* 335 $[M+H]^+$) was observed. Thus, negative-ESI mode was selected for further analyses.

Typical chromatograms for a roots' extract of *S. reticulata* are shown in Fig. 2, demonstrating good baseline separation for all peaks corresponding to **1**, **2**, and dulcitol (**3**). These peaks were identified by comparison of their retention times and mass fragmentation patterns with those of the standards (**1** and **2**) or an authentic sample (**3**).

3.3. Validation of the assay

Some analytical parameters such as linear range and a limit of quantitation of the developed method were evaluated as shown in Table 1. The calibration curve was linear in the range studied (0.5–5.0 μg/mL), showing a correlation coefficients (R^2) of 0.9979 for **1** and 0.9992 for **2**, respectively. The difference in the slope of calibration curves for **1** and **2** were thought to be caused by the different ionization efficiency. Linear regression equations for their calibration curves for **1** and **2** were $y = 525115x + 34981$ and $y = 164713x - 10070$, respectively, where y is the peak area and x is the concentration of the analyte. The detection limits and quantitation limits were estimated to be 0.015 and 0.050 ng for **1**, and 0.030 and 0.10 ng for **2**, respectively, indicating excellent sensitivity of this method. The intra- and inter-day precisions were determined by estimating the corresponding responses five times on the same day and on five different days, respectively. As the result, the relative standard deviation (RSD) values of **1** and **2** were 4.6% and 6.8% for intra-day assays, and 6.7% and 8.5% for inter-day assays, respectively. Accuracy was determined in recovery experiments using a water extract from stems of *S. chinensis*. As shown in Table 2, recovery rates of 85.8–112.6% for **1** and 99.7–106.1% for **2** were obtained, with RSD values of lower than 2.3%.

3.4. Application

According to the protocol established, contents of salacinol (**1**) and kotalanol (**2**) in the roots of *S. reticulata*, *S. oblonga*, and *S. chinensis* were examined. As for *S. chinensis*, those in the stems, leaves, and fruits were also examined (Table 3). The results showed that in the roots' extracts of all the *Salacia* species tested, **1** and **2** were detected in the range of 0.257–0.780% and 0.056–0.156%, respectively. Among the root extracts, one of *S. reticulata* was found to show the highest contents with respect to both **1** (0.780%) and **2** (0.156%). In the stems of *S. chinensis* nearly equal amount of **1** (0.300%) as that in the roots (0.259%) was detected, but with respect to **2**, twice (0.105%) as much as that in the roots (0.056%) was detected. These data were relatively in accordance with their inhibitory activities independently measured (Table 3). In the leaves and fruits of *S. chinensis* were contained scarcely **1** and **2**.

Table 1
Linearities, detection and quantitation limits, and precisions of salacinol (**1**) and kotalanol (**2**).

Analyte	Regression equation[a]	Correlation coefficient (R^2)	Detection limit[b] (ng)	Quantitation limit[b] (ng)	Precision[c] (RSD, %)	
					Intra-day	Inter-day
Salacinol (**1**)	$y = 525115x + 34981$	0.9979	0.015	0.050	4.6	6.7
Kotalanol (**2**)	$y = 164713x - 10070$	0.9992	0.030	0.10	6.8	8.5

[a] In the regression equation, x is the concentration of analyte solution (μg/mL), and y is the peak area of analyte.
[b] Values are the amount of analyte injected on-column.
[c] Precision and accuracy of the analytical method were tested using the water extract from stems of *S. chinensis* ($n = 5$).

Table 2
Recoveries of salacinol (**1**) and kotalanol (**2**).

Add (μg/mL)	Salacinol (**1**)				Kotalanol (**2**)			
	Recovery (%)[a]			RSD (%)	Recovery (%)[a]			RSD (%)
1.0	85.8	88.4	89.8	2.3	104.6	104.7	102.2	1.4
2.0	108.9	107.1	108.7	0.9	99.7	100.8	101.0	0.7
3.0	108.7	108.9	112.6	2.0	106.1	102.7	105.1	1.7

[a] Recoveries of salacinol (**1**) and kotalanol (**2**) spiked with a water extract from stems of *S. chinensis* (500 μg/mL).

Table 3
Contents of salacinol (**1**) and kotalanol (**2**) in *Salacia* species[a].

	Part	Loss on drying (%)[b]	Content[c] (mg/g from dry material)		Extraction yield (%)	Content (% from extract)		Enzyme (substrate: 37 mM)	
			Salacinol (**1**)	Kotalanol (**2**)		Salacinol (**1**)	Kotalanol (**2**)	Sucrase IC_{50} (μg/mL)	Maltase IC_{50} (μg/mL)
S. reticulata	Root	10.4	0.661 ± 0.009	0.132 ± 0.007	8.5	0.780 ± 0.110	0.156 ± 0.008	35.3	95.4
S. oblonga	Root	9.7	0.548 ± 0.018	0.187 ± 0.015	21.3	0.257 ± 0.008	0.088 ± 0.007	61.9	171.4
S. chinensis	Root	8.7	0.490 ± 0.003	0.106 ± 0.006	19.0	0.259 ± 0.002	0.056 ± 0.003	57.9	157.7
	Stem	4.0	0.281 ± 0.012	0.098 ± 0.007	9.4	0.300 ± 0.012	0.105 ± 0.007	36.5	87.3
	Leaf	8.2	0.044 ± 0.011	0.046 ± 0.009	13.5	0.032 ± 0.010	0.034 ± 0.007	>200	>500
	Fruit	11.7	0.084 ± 0.009	0.072 ± 0.018	57.0	0.015 ± 0.007	0.013 ± 0.003	>200	>500

[a] Each powdered sample was extracted two times with H_2O under reflux for 120 min.
[b] Each powdered sample was dried at 105 °C for 8 h.
[c] Values are means ± SD (n = 3).

4. Conclusions

In conclusion, practical method for quantitative determination of potent α-glucosidase inhibitors, salacinol (**1**) and kotalanol (**2**), in *Salacia* species has been developed. The method was validated with respect to linearity, detection limit, precision, and accuracy. The assay was reproducible and precise and could be readily utilized for the quality evaluation of *Salacia* species and related products. Among the several samples tested in the present study, the roots' extract of *S. reticulata* gave the best score with respect to the contents of **1** and **2**. Recently, their desulfonated analogs, neosalacinol [6,10,11] and neokotalanol [6,8,12] have also been reported as another sulfoniums responsible for the activity. Attempts to develop the quantitative analyses of these highly polar constituents for more precise evaluation of *Salacia* species are in progress.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jpba.2010.02.025.

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