J. Pharm. Pharmacol. 1993, 45: 839–841
Communicated December 24, 1992

Liquid chromatographic determination of magnolol in urine collected from volunteers after a single dose of Saiboku-To, an oriental herbal medicine for bronchial asthma

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Abstract—Saiboku-To is an anti-asthmatic herbal remedy which consists of ten herbal extracts. To investigate the clinical relationship between the effects and chemical components of Saiboku-To, a simple and sensitive high-performance liquid chromatographic method (HPLC) for determination of magnolol, one of the major urinary products, was developed. Organic solvent extraction of urinary magnolol was conducted by diatomaceous earth column rapid-flow fractionation using ethanol/dichloromethane (8/92, v/v). Recovery rates of magnolol were more than 99% with coefficient of variations less than 6% in the concentration range 9·7–970 ng mL^{-1}. Subsequent HPLC determination of magnolol was achieved using a conventional silica-gel column, a mobile phase mixture of acetic acid/diethyl ether/*n*-hexane (0·2/17·0/82·8, v/v), and a UV-absorption detector set at 290 nm. Calibration was on the basis of peak height ratio between magnolol and flavone as an internal standard. The method was used to demonstrate excretion profiles of magnolol in healthy and asthmatic subjects following single administration of Saiboku-To.

Magnolol (Fig. 1), is an active component of *Magnolia officinalis* contained in Saiboku-To, which is a traditional Chinese herbal remedy used in patients with bronchial asthma (Nagano et al 1988).

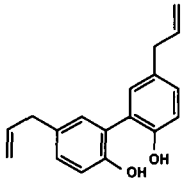

FIG. 1. Chemical structure of magnolol.

Determination of magnolol in *M. officinalis* has been achieved by thin-layer chromatography and gas-liquid chromatography (Fujita et al 1973b). Urinary magnolol concentration following Saiboku-To administration, however, was lower than the reported determination limits. We therefore needed to develop a sensitive and accurate assay method.

Materials and methods

Chemicals and reagents. Organic solvents and reagents of analytical grade were purchased from Wako Pure Chem. Co. (Osaka, Japan). Saiboku-To (TJ-96) consists of brownish granules of the dry extracts and is commercially available from Tsumura Co. (Tokyo, Japan). Magnolol isolated from *M. officinalis* was contributed by Professor Y. Sashida, Tokyo College of Pharmacy (Fujita et al 1973a). Flavone, an internal standard, and beef liver β-D-glucuronidase were obtained from Sigma Chemical Co. (St Louis, MO, USA).

β-D-Glucuronidase treatment of urine samples. Magnolol exists in both free and glucuronic acid conjugated forms in urine (Homma et al 1992). Extraction of the free magnolol was carried out without any pretreatment. Urine samples were treated with beef liver β-D-glucuronidase before extraction of the total magnolol as follows. A 10 μL sample of a beef liver β-D-glucuronidase preparation (55 000 units) was added to 1 mL urine adjusted to pH 5 with acetic acid. The resulting mixture was incubated at 37°C for 12 h and was stored at −20°C until analysis.

Rapid flow fractionation of magnolol. Fractional extraction of urinary magnolol using rapid flow fractionation (RFF) was performed by the general procedures described previously (Oka et al 1984; Homma et al 1991). The apparatus consisted of two glass columns (Kusano Scientific Co., Tokyo, Japan) packed with diatomaceous earth granules of mean particle size 50 μm (Celite No. 545; Johns Manville, Denver, CO, USA). The first column (inner volume 3·2 mL) was for sample extraction and the second column (inner volume 0·8 mL) was for washing the extract to eliminate acidic contaminants. The two columns were connected to each other using a simple Teflon plug (Kusano Scientific Co.).

Seventy microlitres of sodium hydroxide solution (50 g L^{-1}) was injected into the washing column before connecting this column to an outlet of the extraction column. A sample of urine (0·5 mL) containing 40 ng flavone was introduced into the extraction column. The reservoir tube (inner volume 7 mL) was attached to an inlet of the extraction column and filled with the solvent mixture ethanol/dichloromethane (8/92, v/v). The solvent was flushed out of the column system using nitrogen gas at a pressure of 1–2 kg cm^{-2}. The effluent was collected in a glass tube and evaporated to dryness. The residue was reconstituted with 20 μL methanol/diethyl ether (8/92, v/v) for injection onto the HPLC column.

Chromatography conditions. The HPLC system used in this study consisted of a solvent delivery pump (BIP-I, Jasco, Tokyo, Japan), a syringe-loading sample injector (Model 7125, Rheodyne, Cotatti, CA, USA), a silica-gel column (LiChrosorb Si-60, particle size of 5 μm, 4 mm i.d. × 250 mm; Merck, Darmstadt, Germany), a UV-detector (Uvidec 100-V, Jasco), and a single pen recorder (Pantos-U 228, Tokyo Denshi, Tokyo, Japan). The wavelength of the detector was set at 290 nm and sensitivities, 0·02–0·0025 aufs. The mobile phase was acetic acid/diethyl ether/*n*-hexane (0·2/17·0/82·8, v/v) at a flow rate of 1·5 mL min^{-1}.

Subjects and sample collection. Seven healthy male volunteers, aged 23·3 ± 2·1 years, and ten asthmatic patients, aged 48·4 ± 16·5 years, participated in the study. Informed consent was obtained from each subject and the study was approved by the Ethics Committee of the Tokyo Medical College Hospital. Medication and food were not controlled. The patients had been treated by co-administrations of bronchodilators such as theophylline and β-stimulants, oral and inhaled corticosteroids, and anti-allergic drugs. Urine was collected at 1, 3, 6, 9, and 12 h intervals after a single administration of Saiboku-To at a dose of 5 g. These samples were stored at −20°C until analysis.

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Calculations. Elimination half-lives ($t_{\frac{1}{2}}$) were calculated from the individual excretion rates in accordance with a one-compartment pharmacokinetic model. The data including urinary magnolol concentrations, excretion rates, $t_{\frac{1}{2}}$, and amounts of excretion are presented as mean ± standard deviation. The differences in the data between the subject groups were analysed using the unpaired Student's *t*-test.

Results

Recovery of magnolol from urine. Table 1 shows extraction recovery rates of magnolol added to urine at known concentrations (9·7, 97, and 970 ng mL^{-1}). Variations of recovery rates at each concentration were obtained with five consecutive analyses. The rates were higher than 99% in every case with the coefficients of variation less than 6%. Recovery rates of flavone, an internal standard, were also determined at the concentration of 80 ng mL^{-1}, the average value being 103·3 ± 2·9% with a coefficient of variation of 2·8%. These results showed that our method of fractional extraction of magnolol was successfully optimized.

Table 1. Recovery of magnolol from urine.

Concn of magnolol added (ng mL^{-1})	Recovery (%)	Coeff of variation (%)
9·7	100·6 ± 3·6	3·6
97·0	99·0 ± 5·8	5·9
970·0	99·9 ± 3·6	3·6

Mean ± s.d.

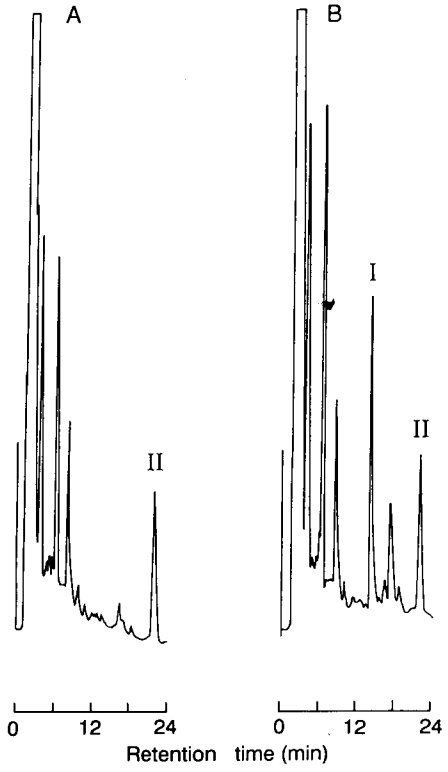

FIG. 2. Typical chromatograms for determination of urinary magnolol. I magnolol, II flavone as the internal standard. Before (A), and 1 h after (B) the administration of Saiboku-To. Calculated concentration of magnolol in the chromatogram shown was 277·7 ng mL^{-1}.

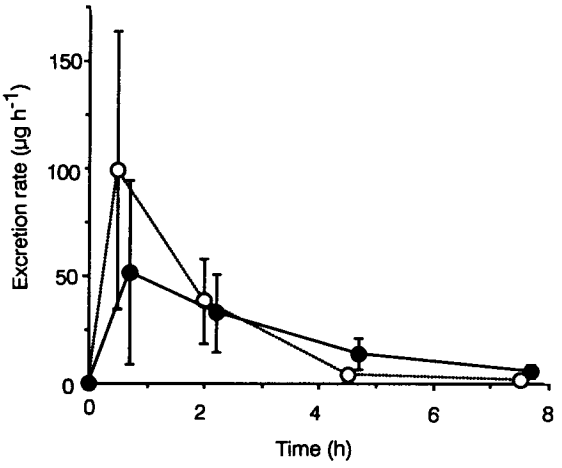

FIG. 3. Urinary excretion rate of magnolol after a single administration of 5 g Saiboku-To to healthy (○) and asthmatic (●) subjects.

Chromatography. Typical chromatograms before and after the administration of Saiboku-To are illustrated in Fig. 2. Capacity ratio (k′ = (retention volume/hold-up volume) − 1) of magnolol was 9·0 appearing at 13·6 min. No interference peak was observed at the same retention time as magnolol. The detection limit of magnolol was 2·5 ng injected with a signal to noise ratio of 3.

Calibration curve. Concentration-peak height ratio of magnolol was examined over the range 10–10 000 ng mL^{-1} giving a regression equation of

$$Y = 7{\cdot}45 \times 10^{-3} X - 6{\cdot}91 \times 10^{-2}$$

where X is the concentration of magnolol (ng mL^{-1}) and Y the peak-height ratio of magnolol against flavone. The correlation coefficient (r) was 0·9989.

Urinary excretion profiles of magnolol in healthy and asthmatic subjects. Changes in urinary excretion rates of magnolol after the administration of Saiboku-To at a single dose of 5 g in both healthy subjects and patients are illustrated in Fig. 3. The highest excretion rate of magnolol was observed in the healthy subjects at 1 h after administration. Although the asthmatic patients showed the highest rates at 1 h, the value was less than that of the healthy subjects. The calculated value of $t_{\frac{1}{2}}$ of the patients was 1·40 ± 0·47 h which was longer than that of the healthy subjects (0·93 ± 0·13 h). This difference in $t_{\frac{1}{2}}$ was statistically significant ($P < 0{\cdot}05$).

Excretion of magnolol was observed until 9 h after administration. Cumulative amounts of the free and conjugated magnolol excreted in urine for 9 h is shown in Table 2. Around 95% of urinary magnolol existed in the glucuronic acid-conjugated form in both healthy and asthmatic subjects. No significant differ-

Table 2. Cumulative amounts of urinary magnolol in healthy subjects and asthmatic patients for 9 h after a single administration of 5 g Saiboku-To.

	Excreted amounts (μg)		
	Free	Conjugated	Total
Healthy subjects (n = 7)	4·4 ± 1·8	187·9 ± 88·6	192·3 ± 88·8
Asthmatic patients (n = 10)	5·3 ± 4·3	177·3 ± 62·7	175·9 ± 68·1

Mean ± s.d.

ences were observed in the conjugation rate of magnolol between the groups.

Discussion

In a previous study, we identified three major components in the urine of patients receiving Saiboku-To treatment (Homma et al 1992) as magnolol, dihydroxydihydro-magnolol, and liquiritigenin. We expected these components to play an important role for corticosteroid-sparing effects on asthmatic patients. Our particular interest was focused on magnolol because this component showed inhibitory activity against 11β-hydroxysteroid dehydrogenase (unpublished data).

We improved our original extraction procedure and added an HPLC stage for quantitation of magnolol in urine. Thus, we were able to determine magnolol with an improved detection limit of 2·5 ng. Sensitivity and specificity of this method are comparable with those of LC-MS methods using radiolabelled magnolol (Hattori et al 1986). We applied our method to determination of the free and total magnolol after a single administration of Saiboku-To as shown in Table 2.

A single dose of 5 g Saiboku-To contained 2·1 mg magnolol. Around 10% of the dosed magnolol was excreted in the urine collected for 9 h following administration. This recovery rate was comparable with that obtained in animal experiments (Hattori et al 1986). Individual variations of magnolol excretions were observed at the same single dose of Saiboku-To per subject, which could not be explained by body weight differences. Extended studies employing our method and patient groups such as responders and non-responders under long-term Saiboku-To treatment will be useful in determining the clinical implications of magnolol.

This work was supported by the Ministry of Education in Japan (Grant in Aid No. 03857345). We are grateful to Professor Y. Sashida for excellent discussions and to Mr T. Yamada and Ms C. Higuchi for their technical assistance.

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J. Pharm. Pharmacol. 1993, 45: 841–843
Communicated October 16, 1992

DAU 6215, a novel 5-HT$_3$-receptor antagonist, selectively antagonizes scopolamine-induced deficit in a passive-avoidance task, but not scopolamine-induced hypermotility in rats

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Abstract—This study examined the effects of DAU 6215, a selective 5-HT$_3$-receptor antagonist, on either impairment of a passive-avoidance task or hypermotility, both caused by scopolamine in rats. In the first experiment, scopolamine (0·75 mg kg^{-1}, i.p.) disrupted acquisition of a one-trial 'step through' passive-avoidance response. Pretreatment with DAU 6215 (1, 10, 30 and 100 μg kg^{-1}, i.p.) antagonized this deficit induced by scopolamine, with a bell-shaped dose-response curve. Scopolamine (0·75 mg kg^{-1}, i.p.) produced a significant increase in locomotor activity which was unaffected by pretreatment with DAU 6215 (10 and 30 μg kg^{-1}, i.p.). The present results further support the suggestion that 5-HT$_3$-receptor antagonists may prevent the memory disturbance caused by a reduction in central cholinergic function in the rat. The inefficacy shown by DAU 6215 on hyperactivity induced by scopolamine appears to rule out the possibility of a pharmacokinetic interference between DAU 6215 and scopolamine.

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There is ample evidence indicating that 5-hydroxytryptamine (5-HT) mechanisms play a significant role in learning and memory processes (McEntee & Crook 1991). Stimulation of the 5-HT-ergic nervous system has a negative influence on learning and memory (Fibiger et al 1978), while 5-HT-ergic antagonists produce an enhancement of cognitive functions (Altman & Normile 1986).

Among the compounds acting on the different 5-HT-receptor subtypes, 5-HT$_3$-receptor antagonists have been reported to enhance learning and memory performance in animals (Barnes et al 1990; Chugh et al 1991a, b).

Results from these studies demonstrated that 5-HT$_3$-receptor antagonists inhibited the impairment in performance caused by cholinergic deficits in a passive avoidance task in mice (Chugh et al 1991a) and in a working memory task in rats (Barnes et al 1990).

The validation of laboratory tests, or animal models, designed to investigate cognition enhancing drugs is far from established