Simultaneous determination of rhynchophylline, isorhynchophylline, and their eight metabolites in rats

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Abstract

A quantification method using HPLC-MS for simultaneous determination of two major bioactive alkaloid isomers [rhynchophylline (RHY) and isorhynchophylline (ISOR)] and their 8 metabolites were established. The method provided satisfactory precision, accuracy and sensitivity in quantification of these compounds in biorea. The quantification limit was as low as 0.01ng/ml. With this method, the pharmacokinetic profiles of these compounds were investigated after oral administration of two Kampò formulations yokukansan and chotosan, and compared with those after oral administration of pure compounds. Interestingly, the contents of RHY and ISOR in rat brain after administration of extracts of yokukansan and chotosan were higher than those in rat brain after individual administration of RHY and ISOR, respectively.

Key words chotosan, yokukansan, metabolism, rhynchophylline, isorhynchophylline.

Abbreviations API-ESI, atmospheric pressure interface-electrospray ionization; BDC, bile duct-cannulated; CTS, chotosan; DMSO, dimethyl sulfoxide; ISOR, isorhynchophylline; RHY, rhynchophylline; RSD, relative standard deviation; SIM, selective ion monitoring; TIM, total ion monitoring; UDPGA, uridine 5′-diphosphogluconic acid; YKS, yokukansan.

Introduction

Chotosan (CTS) and yokukansan (YKS) are two well-known prescriptions used in Kampò medicine (traditional Sino-Japanese medicine) to treat hypertension, headache, insomnia, and tinnitus. Recently CTS was reported to enhance macrophage colony-stimulating factor mRNA expression in the ischemic rat brain and C6Bu-1 glioma cells, and to ameliorate chronic cerebral hypoperfusion-induced deficits in object recognition behaviors and central cholinergic systems in mice. YKS was also found to inhibit social isolation-induced aggression, methamphetamine-induced hyperlocomotion in rodents, and development of atopic dermatitis-like lesions in isolated NC/Nga mice, and seems to be useful for prevention or cure of abnormal glutamate release. Uncaria (U.) species are the main component herbs of CTS and YKS and alkaloids, rhynchophylline (RHY) and isorhynchophylline (ISOR) in U. species were suggested to be bioactive compounds, which account for antiamnesia effects of CTS. Recently, RHY was also reported to have antiepileptic effects in kainic acid-induced seizures.

We recently studied the metabolism of pure RHY and ISOR alkaloids in rats and found that RHY and ISOR were hydroxylated into 11-hydroxyrhynchophylline (M3a), 11-hydroxyisorhynchophylline (M3b), 10-hydroxyrhynchophylline (M4a), and 10-hydroxyisorhynchophylline (M4b) in phase I metabolism, then which were
subsequently conjugated with glucuronic acid to form 11-hydroxyrhynochophylline 11-O-β-D-glucuronide (M1a), 11-hydroxyisorhynochophylline 11-O-β-D-glucuronide (M1b), 10-hydroxyrhynochophylline 10-O-β-D-glucuronide (M2a), and 10-hydroxyisorhynochophylline 10-O-β-D-glucuronide (M2b) in phase II metabolism, respectively. It was confirmed that RHY was transformed to M3a and M4a, while ISOR to M3b and M4b, when incubated with rat liver microsomes. On the other hand, the former was transformed to M1a and M2a, and the latter to M1b and M2b, respectively, when incubated together with uridine 5′-diphosphoglucuronic acid (UDPGA) and rat liver microsomes in vitro.9,10

The present study was carried out to establish a quantification method to determine simultaneously two isomeric alkaloids RHY and ISOR and their 8 metabolites (see Fig. 1) after oral administration of extracts of two Kampo formulations. Comparisons of the pharma-

![Fig. 1 Structures of RHY, ISOR, and metabolites (M1a, M2a, M3a, M4a, M1b, M2b, M3b, and M4b)]
cokineal profiles of these compounds after administration of the Kampo formulations with those after administration of the respective pure compounds are also discussed.

Materials and Methods

Enzymes and chemicals: β-Glucuronidase (EC No. 2.3.1.31, Type B-1), heparin sodium salt, taurocholic acid sodium salt, and other chemicals were purchased from Sigma (St. Louis MO, USA). Pentobarbital sodium (64.8 mg/ml) was purchased from Kyoritsu Seiyaku Co., Tokyo, Japan. Saline (0.9%) was obtained from Otsuka Pharmaceutical Co. Ltd., Tokyo, Japan. Elution solvents for LC-MS were of HPLC grade. RHY, ISOR, M1a-M4a and M1b-M4b were isolated and their structures were determined as reported previously. All of these oxindole alkaloids were kept at 4 °C until use. We used RHY and ISOR at > 99.5% purity confirmed by HPLC for all experiments.

Chotosan and yokukansan: All of the medicinal herbs and mineral were purchased from Tochimoto Tenkaido Co., Osaka, Japan. Chotosan formulation1 comprises dried branches (15 g, cut to ca. 1 cm in length) with hooks of U. rhytchophylla Miquel and U. sinensis Haviland (Rubiaceae) in a ratio of 1:2 (wt/wt), dried flowers (15 g) of Chrysanthemum indicum L. (Compositae), dried roots (15 g, sliced to ca. 0.5 cm in diameter) of Saposhnikovia divaricata Schischkin (Umbelliferae), powdered gypsum (30 g, CaSO4·2H2O), roots (15 g, sliced to ca. 0.5 cm in diameter) of Codonopsis species (dangshen in Chinese traditional medicine), dried big roots (15 g, cut to ca. 0.5 cm in diameter) of Ophiopogon japonicus Ker-Gawler (Liliaceae), dried sclerotium (15 g, outer layer stripped, cut to ca. 0.5 cm in diameter) of Poria cocos Wolf (Polyporaceae), dried roots (15 g, cork layer stripped, sliced to ca. 0.5 cm in diameter) of Pinellia ternata Breitenbach (Araceae), dried fruit peel (15 g, cut to ca. 0.5 cm in diameter) of Citrus unshiu Markovich (Rutaceae), dried roots (6 g, cut to ca. 0.5 cm in length) of Glycyrrhiza species, and dried roots (6 g, cut to ca. 0.5 cm in diameter) of Zingiber officinale Roscoe (Zingiberaceae). Yokukansan prescription1 includes branches of U. species (3 g), sclerotium of Poria cocos Wolf (4 g), roots of Glycyrrhiza species (2 g) as described in chotosan, dried roots (2 g, cut to ca. 1 cm in length) of Bupleurum falcatum L. (Umbelliferae), dried roots (3 g, cut to ca. 0.5 cm in diameter after blanched) of Angelica acutiloba Kitagawa (Umbelliferae), dried roots (3 g, cut to ca. 0.5 cm in diameter after blanched) of Cnidium officinale Makino (Umbelliferae), and dried roots (4 g, cut to ca. 0.5 cm in diameter) of Atractylodes japonica Koidzumi ex Kitamura (Compositae).

Preparation of a decoction of chotosan: Gypsum was boiled in 200 ml of water for 15 min. All of the others except dried flowers (15 g) of Chrysanthemum indicum were added into a gypsum solution with water (up to 500 ml) and macerated for 20 min. After the mixture was boiled for 30 min, dried flowers of Chrysanthemum indicum were added and the boiling continued for another 10 min. The hot mixture was decanted to leave a residue, which was boiled again in 500 ml of water for 40 min. After passed through purified cotton to eliminate coarse debris, the hot decoction was cooled to room temperature. The pooled decoction (n = 3, ambient) was extracted with a same volume of ethyl acetate in triplicate. The extract was concentrated and lyophilized to give a residue (CTS). Portions (0.98 g) of the residue (yellow brown solid) was dissolved in 2.0 ml of DMSO to yield a clear brown solution (3.0 ml), which was kept at -22 °C until analysis or use for animal experiments.

Preparation of a decoction of yokukansan: After all components except branches of U. species were dipped in 500 ml of water for 20 min and boiled for 20 min, branches of U. species (15 g) were added and boiled for another 20 min. The residue decanted from the hot mixture was boiled again in 500 ml of water for 40 min. The coarse debris in the hot decoction was eliminated by filtration through purified cotton and the filtrate was cooled to room temperature. The pooled filtrate (n = 3, ambient) was extracted with an equal volume of ethyl acetate in triplicate. The extract was concentrated and lyophilized to give a residue (YKS). Portions (0.83 g each) of the residue (yellow brown solid) was dissolved in 1.7 ml of DMSO to yield a clear brown solution (2.4 ml), which was kept at -22 °C until analysis or use for animal experiments.
CTS and YKS are highly hygroscopic, but readily soluble in water at 37-38 °C. The exactly weighted extracts were immediately dissolved in DMSO.

**Animals and administration of CTS and YKS extracts and pure compounds:** Male Wistar rats (7 weeks) were procured from Sankyo Labo Service Corporation, Inc., Tokyo, Japan. All animal care and experiments proceeded at the animal experimental center of the University of Toyama in accordance with the guidelines provided by the Life Science Research Center of the same institution. The animals were fed in the public husbandry room of the center for intact rats for 1 week, and acclimated for 3 d in metabolic cages with 12-hour-light intervals in the metabolic room. Rats were housed in the metabolic cages, supplied standard chow and drinking water, which had been sterilized at 121 °C for 15 min. Oral dosages to rats (250 g prior to administration) was 0.294 g of CTS (containing 6.21 mg of RHY and 4.16 mg of ISOR), 0.311 g of YKS (containing 5.98 mg of RHY and 4.06 mg of ISOR), and 9.4 mg each pure RHY\(^9\) and ISOR\(^10\) in DMSO with a total volume of 0.90 ml, respectively. These doses do not refer to the maximal tolerated dose in rats. The animals were fasted overnight with free access to drinking water and 3 h after administration. Oral administration of CTS, YKS and pure compounds to rats was conducted with a gavage tube (KN-348, φ1.2 × L 80 mm, Natsume Seisakusho Co. Ltd., Tokyo, Japan). No appreciable side effects were observed in rats throughout the experiments.

**Preparation of plasma samples after oral administration:** The rats were anesthetized by intraperitoneal (i.p.) injection of 12 mg of pentobarbital sodium before collecting blood samples. Plasma was separated from blood (6 - 8 ml) collected from the inferior vena cava of the rats with a heparinized syringe at 30 min, 1, 2, 3, 4, 6, 8, 12, 24, 36, 48, 72 and 96 h after administration, followed by centrifugation at 5100 × g (Kubota 6800 refrigerated centrifuge, Kubota Co., Tokyo, Japan) for 15 min at 4 °C to produce a supernatant (plasma, about half-volume of the blood). The plasma (2.0-10.0 μl) was directly analyzed by LC-MS. Another 1.0 ml of the plasma was treated with β-glucuronidase (32,000 Sigma units)\(^9\) and analyzed by LC-MS.

**Bile collection and preparation of bile samples after oral administration:** The common bile duct of a rat was cannulated with polyethylene tube\(^9-11\) (medical grade, PE 10: i.d. 0.28 × o.d. 0.61 mm, or PE 50: i.d. 0.58 × o.d. 0.96 mm) predose. The bile sample (0.6-0.7 ml/h, clear yellow-green liquid) was collected at an 1-h interval for continuous 16 h after a bile duct-cannulated (BDC) rat was orally administered CTS or YKS. At the same time the rat received an infusion of the artificial biliary solution (100 mM sodium taurocholate and 0.5% potassium chloride in 0.9% saline) through its duodenum at 0.5 ml/h.\(^12\)

A container for bile accumulation was cooled with ice during sampling, and bile samples were kept at -22 °C until analysis or enzyme-treatment.

The bile sample (2.0-5.0 μl) collected at an 1-h interval was analyzed by LC-MS. The rest bile sample was digested with β-glucuronidase (20000 Sigma units) and analyzed by the same methods as reported previously.\(^9\)

**Preparation of urinary and fecal samples after oral administration:** Rat urinary (15 - 21 ml over a period of 24 h), and fecal (15 - 20 g over 24 h) samples were collected at 24-h intervals for 4 days after oral administration of extracts to intact rats. The urinary containers were kept in ice bath during collection. All of the urinary and fecal samples were kept at -22 °C until enzyme-treatment and analysis. Urinary samples (2.0-5.0 μl) were directly analyzed by LC-MS. The lyophilized residue of urinary samples (10.0 ml) was digested with β-glucuronidase (60,000 Sigma units) and analyzed by the same methods.\(^9\)

All of the fecal sample collected in every 24-hour interval was ground and sonicated in ethyl acetate (200 ml × 3) for 10 min. The extracts were pooled and concentrated under vacuum at room temperature to yield a yellow residue, which was dissolved in methanol and analyzed by LC-MS.

**Preparation of brain samples:** Each rat was anesthetized by intraperitoneal injection with 26 mg of a pentobarbital sodium 3 h after oral administration of either CTS or YKS. The brain including the cerebrum, cerebellum and about 0.5 cm of medulla oblongata was removed en bloc after decapitation. Traces of blood on the brain surface were washed with saline.\(^9,13\) Whole brains
were lyophilized, ground and sonicated in methanol (5 ml × 3) for 5 min. The mixture was separated by centrifugation at 2220 × g for 5 min. The supernatant was pooled and concentrated under vacuum at room temperature to afford a white residue, which was dissolved in methanol (8.0 ml), passed through a 0.45 μm filter and analyzed by LC-MS.

**Controls:** Control rat blood, bile, urine, feces and brains were collected after oral administration of a DMSO vehicle and processed as described above.

**LC-ESI-Ion trap MS**<sup>a</sup> **measurements:** Both in vivo and in vitro samples were analyzed using an Agilent 1100 series HPLC system coupled with a Bruker Daltonics<sup>®</sup> Esquire 3000 plus mass spectrometer. An integrated LC-MS control and data were processed using the Agilent ChemStation for the LC-MS system and Bruker Daltonics Esquire 5.1 (Esquire Control Version 5.1, and DataAnalysis Version 3.1).

The samples were applied to a Cosmosil packed column (5C18-MS-II, 4.6 i.d. × 150 mm) at 30 °C, and UV detection was set at 245, 254, 230, 208 and 280 nm. The elution system comprised an increase of solvent B (0.01% v/v acetic acid in CH<sub>3</sub>CN) from 10 to 30% in solvent A (0.01% v/v acetic acid) within 40 min, then to 100% within 20 min at a flow rate of 1.0 ml/min. All samples were passed through a 0.45 μm filter before analysis. Through a splitting device, 20% of the eluate from a diode array detector was introduced to the atmospheric pressure interface-electrospray ionization (API-ESI) for total ion monitoring (TIM) or selective ion monitoring (SIM). The fragmentation cut-off was set at 27% of the precursor mass. The scan range of the ion trap was from m/z 50 to 1,000 in the positive ion mode, the nebulizer was set at 50 psi, dry gas at 10.0 l/min and the dry temperature was 360 °C.

Quantitative determination of RHY, ISOR, and their metabolites was performed by LC-MS analysis.<sup>9,10</sup> Calibration curves of RHY, ISOR, and their metabolites were plotted on the peak areas in EIC against their known concentrations in methanol, separately. The correlation coefficient r<sup>2</sup> was more than 0.999 in a concentration range from 4.0 × 10<sup>-10</sup> to 4.0 × 10<sup>-7</sup> mol/l.

**Recovery experiments:** The recovery of RHY, ISOR, and their metabolites was examined by adding exact amounts of the compounds or their metabolites (4.0 × 10<sup>-10</sup>, 1.2 × 10<sup>-8</sup>, and 4.0 × 10<sup>-7</sup> mol/l, respectively) to body fluids (6.0 ml blood from inferior vena cava, and 0.6 ml bile), excretions (20.0 ml urine, and 20.0 g feces),<sup>9</sup> and brain (0.400 g, dry weight), and then measuring the respective concentrations by LC-MS. The recoveries from five analyses varied from 95.2 to 102.5%.

**Precision experiments:** The precision of the analysis of RHY, ISOR, and their metabolites was examined by comparing the results of intra- and inter-day assays with relative standard deviation (RSD) values. The RSD values for intra-day (5 measurements on the same day for each sample) and inter-day (5 measurements per day for each sample for 5 continuous days) assay were both < 5.0%.

All of the data in Figs. 3, 4, 6, and 7, are shown as means ± S.D. (n = 3), unless otherwise indicated.

**Results and Discussion**

**Stability of CTS and YKS extracts including oxindole alkaloids:** The chemical constituents of CTS and YKS dissolved in DMSO did not appreciably changed (e.g. isomerization) during 6 months at -22 °C, when analyzed by LC-MS. The sealed solid extracts (filled with N<sub>2</sub>) were stable for 6 months at 4 °C. RHY and ISOR were detected by LC-MS with a sensitive response after CTS and YKS were dissolved in methanol.

**RHY and ISOR in the circulation system:** After oral administration of CTS and YKS to rats, RHY and ISOR (ESI-MS m/z: 385 [M+H]<sup>+</sup>) were detected as free forms in plasma at retention times 20.6 and 19.2 min, respectively (Fig. 2). These compounds were identified by comparing the retention times and MS spectra including MS1/MS and MS3 with those of the authentic samples (Fig. 2 and Table 1). Both RHY and ISOR showed the maximum plasma concentrations 3 h (observed values) after administration, and decreased appreciably within 12 h (Fig. 3a and 3b, respectively). No enterohepatic circulation of RHY and ISOR was observed in this experiment (Fig. 3). In rat plasma, no metabolites of RHY and ISOR were detected even in trace.
Fig. 2 LC-MS (EIC) elution profiles of rat plasma taken 3 h after oral administration of CTS (a), ISOR (b), and RHY (c), respectively, monitored at m/z 385 ± 0.5 and mass spectra of ISOR (d) and RHY (e). Similar elution profiles were obtained after administration of YKS, but RHY and ISOR appeared in lower concentrations than those after administration of CTS.
Table 1  Retention times and major mass spectral data in positive ion mode of the two alkaloids and their metabolites

<table>
<thead>
<tr>
<th>Compound</th>
<th>Retention time (min)</th>
<th>Mass spectral data (m/z)</th>
<th>MS</th>
<th>MS/MS</th>
<th>MS'&lt;sup&gt;1&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>M1b</td>
<td>8.8</td>
<td></td>
<td>577</td>
<td>401</td>
<td>369, 337</td>
</tr>
<tr>
<td>M2b</td>
<td>9.6</td>
<td></td>
<td>577</td>
<td>401</td>
<td>369, 337</td>
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<tr>
<td>M3b</td>
<td>9.3</td>
<td></td>
<td>401</td>
<td>369, 337</td>
<td>337, 281</td>
</tr>
<tr>
<td>M4b</td>
<td>10.1</td>
<td></td>
<td>401</td>
<td>369, 337</td>
<td>337, 281</td>
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<td>M1a</td>
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<td></td>
<td>577</td>
<td>401</td>
<td>369, 337</td>
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<td>577</td>
<td>401</td>
<td>369, 337</td>
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<td>M3a</td>
<td>11.0</td>
<td></td>
<td>401</td>
<td>369, 337</td>
<td>337, 281</td>
</tr>
<tr>
<td>M4a</td>
<td>12.9</td>
<td></td>
<td>401</td>
<td>369, 337</td>
<td>337, 281</td>
</tr>
<tr>
<td>ISOR</td>
<td>19.2</td>
<td></td>
<td>385</td>
<td>353</td>
<td>321, 269</td>
</tr>
<tr>
<td>RHY</td>
<td>20.6</td>
<td></td>
<td>385</td>
<td>353</td>
<td>321, 269</td>
</tr>
</tbody>
</table>

Main fragments are listed.

Fig. 3  Time curves of plasma-concentrations of RHY (a) and ISOR (b) after oral administration of CTS and YKS separately. CTS and YKS were orally administered at dose of 0.294 and 0.311 g, respectively, to rats.
The concentrations of RHY and ISOR in rat plasma after oral administration of CTS and YKS were lower than those after administration of pure RHY and ISOR (Fig. 4). This may be due to the competition among all of the constituents in the extracts of two formulations in the absorption process.\(^2\)\(^9\)

**RHY, ISOR, and their metabolites in rat bile:** After oral administration of CTS to BDC rats, metabolites **M1a, M1b, M2a** and **M2b** (ESI-MS m/z: 577 [M+H]) together with RHY and ISOR were detected in rat bile (Fig. 5a). A similar elution profile was obtained after oral administration of YKS but **M1a, M1b, M2a**, and **M2b** appeared in lower concentrations than those after administration of CTS. Figures 6 and 7 show the accumulated concentrations of RHY and ISOR verses times after administration in rat bile, and the time courses of **M1a, M1b, M2a**, and **M2b** (7a, administration of CTS; 7b, administration of YKS). The respective LC-MS elution profiles of bile samples after oral administration of RHY and ISOR to BDC rats (Fig. 5b and 5c) confirmed that **M1a** and **M2a** were originated from RHY, while **M1b** and **M2b** from ISOR. These four metabolites did not flow into the circulation system of rats, but excreted directly in to the intestinal tract, when monitored by LC-MS. All of the metabolites had the same quasi-molecular ion peak at m/z 577 ([M+H]) in the ESI-MS spectra (Fig. 5d-g; Table 1), but different retention times (8.8, 9.6, 10.5 and 12.5 min). The LC-MS elution profiles of β-glucuronidase-treated bile samples after oral administration of CTS and YKS show that **M1a, M1b, M2a** and **M2b** were quantitatively hydrolyzed into **M3a, M3b, M4a** and **M4b** (Fig. 8, Table 1), similar to the case of independent administration of pure compounds RHY and ISOR.\(^9\)\(^10\)

About 2.0% of RHY and ISOR containing in CTS and YKS administered were excreted into bile within 16 h, during which 30% of RHY and ISOR were transformed to 11-glucuronides **M1a** and **M1b**, and 10-glucuronides **M2a** and **M2b**, respectively (see Fig. 1), both with a ratio of 1.3:1. After oral administration of the extracts of two formulations CTS and YKS, the concentrations of RHY and ISOR detected in rat bile were lower than those after oral administration of the individual compounds. This finding in bile resembled that found in plasma.

**RHY, ISOR, and their metabolites in urine and feces:** In rat urine and feces, RHY, ISOR, their phase I metabolites **M3a, M3b, M4a** and **M4b** were identified by LC-MS, which were identical to those detected in enzyme-treated bile samples after oral administration of CTS and YKS to BDC rats. Metabolites **M3b** and **M4b** were in unconjugated forms, similar to their isomeric metabolites **M3a** and **M4a** obtained after oral administration of RHY to rats.\(^9\)\(^10\) Tables 2 and 3 show the
Fig. 5  LC-MS (EIC) elution profiles of a rat bile sample collected 3-4 h after oral administration of CTS (a), ISOR (b), and RHY (c), monitored at $m/z$ 577 ± 0.5; and mass spectra of M1b (d), M2b (e), M1a (f), and M2a (g). Similar elution profiles achieved after administration of YKS, but these compounds appeared in lower concentrations than those after administration of CTS.
Fig. 6 Accumulation curves of RHY and ISOR in BDC rat bile after oral administration of CTS and YKS separately.

Fig. 7 M1a, M1b, M2a, and M2b detected in BDC rat bile in 16 h after oral administration of CTS (a) and YKS (b) separately.
- M1a; • M1b; ● M2a; ○ M2b.
Fig. 8 LC-MS (EIC) elution profiles of β-glucuronidase-treated rat bile samples collected 3-4 h after oral administration of CTS (a), ISOR (b), and RHY (c), monitored at m/z 401 ± 0.5 and mass spectra of M3b (d), M4b (e), M3a (f), and M4a (g). Similar elution profiles were obtained after administration of YKS, but these compounds appeared in lower concentrations than those after administration of CTS.
Table 2  Mean concentrations of M3a, M3b, M4a, and M4b excreted into rat urine and feces after oral administration of CTS (n = 3)

<table>
<thead>
<tr>
<th>Time range (h)</th>
<th>In rat urine (µg/ml)</th>
<th>In rat feces (µg/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>M3a</td>
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<tr>
<td>0-24</td>
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<td>0.22</td>
</tr>
<tr>
<td>48-72</td>
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</table>

Table 3  Mean concentrations of M3a, M3b, M4a, and M4b excreted into rat urine and feces after oral administration of YKS (n = 3)

<table>
<thead>
<tr>
<th>Time range (h)</th>
<th>In rat urine (µg/ml)</th>
<th>In rat feces (µg/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>M3a</td>
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<td>0-24</td>
<td>2.32</td>
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<td>0.13</td>
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<tr>
<td>48-72</td>
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</table>

Fig. 9  Mean concentrations of RHY and ISOR excreted in rat urine and feces after oral administration of CTS and YKS separately (n = 3)

A: RHY in rat feces after administration of CTS, B: RHY in rat feces after administration of YKS, C: RHY in rat urine after administration of CTS, D: RHY in rat urine after administration of YKS, E: ISOR in rat feces after administration of CTS, F: ISOR in rat feces after administration of YKS, G: ISOR in rat urine after administration of CTS, H: ISOR in rat urine after administration of YKS; conc. (µg/ml) and conc. (µg/g) for compounds in urine and feces, respectively.
mean concentrations of M3a, M3b, M4a and M4b excreted into rat urine and feces after oral administration of CTS and YKS, respectively. Figure 9 shows the excretion of RHY and ISOR in rat urine and feces, respectively. RHY and ISOR excreted into rat feces (20.0 g, 24 h after administration) were approximately 5-fold of those excreted into urine (20.0 ml, 24 h after administration) regardless of CTS or YKS administration. Twenty-four hours after administration, total amounts of 76.6 and 59.2% of RHY and 88.4 and 71.6% of ISOR were excreted into rat urine and feces after oral administration of CTS and YKS, respectively. RHY (8.2% and 7.7% in CTS and YKS, respectively) and ISOR (10.4% and 9.1% in CTS and YKS, respectively) were converted to the corresponding metabolites M3a, M3b, M4a and M4b after oral administration of CTS and YKS. The relative ratio of M3b to M4b was 3:1.

After oral administration of pure RHY or ISOR to rats, the relative ratio of M3a to M4a or M3b to M4b was 1:1. However, as mentioned above, the ratio of M3b to M4b after oral administration of the extracts of formulations CTS and YKS was 3:1. This reflects that the liver enzymes might have more selective hydroxylation at C-10 than at C-11 of ISOR, when CTS or YKS was administered, although this phenomenon was not detected by in vitro incubation with pure ISOR and rat liver microsomes (data not shown).

**Distribution of ISOR and RHY in rat brains after administration of CTS and YKS:** In rat brain, RHY and ISOR were identified by LC-MS (Fig. 10) after oral administration of CTS or YKS, which shows that these alkaloids could pass through the blood-brain barrier to reach the central nervous system. However, no metabolites from RHY and ISOR were detectable in the brain sample.

Interestingly, the amounts of RHY and ISOR detected in rat brain after oral administration of CTS and YKS were 9-fold and 3-fold higher than those when pure RHY and ISOR administered orally, respectively (Table 4). It is likely that the RHY and ISOR in the extract of these formulations might have stronger penetration power through the blood-brain barrier (BBB) than the pure compounds. As RHY and ISOR are known to have beneficial effects on the central nervous system, higher concentrations of these alkaloids in brain when administered as Kampo formulations may have significant implication on the importance of Kampo formulations, and it is worthy of further investigation on how other components in the formulations may influence the penetration power of these bioactive alkaloids to BBB.

In conclusion, a sensitive and accurate LC-MS

**Table 4:** Amounts of RHY and ISOR detected in rat brains after oral administration of CTS, YKS, RHY and ISOR on the dry weight basis

<table>
<thead>
<tr>
<th>Dose</th>
<th>CTS</th>
<th>YKS</th>
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<tbody>
<tr>
<td></td>
<td>equ. 6.2 mg RHY 4.2 mg ISOR</td>
<td>equ. 6.2 mg RHY 4.2 mg ISOR</td>
</tr>
<tr>
<td>RHY</td>
<td>1.29 ± 0.02</td>
<td>1.02 ± 0.05</td>
</tr>
<tr>
<td>ISOR</td>
<td>0.40 ± 0.03</td>
<td>0.38 ± 0.02</td>
</tr>
</tbody>
</table>

Data shown as mean ± S.D. (n = 3), ND: not detected

**Fig. 10** LC-MS (EIC) elution profiles of a rat brain sample isolated 3 h after oral administration of CTS, monitored at m/z 385 ± 0.5.
method was established for simultaneous quantification of ten alkaloids, such as RHY, ISOR and 8 metabolites, in complex biomedia after oral administration of two Kampo formulations containing Uncaria species. RHY and ISOR were detected in rat plasma, bile, urine, and feces after oral administration of CTS and YKS. No metabolites were detected in rat plasma by LC-MS, whereas 10- and 11-hydroxymethylalcohols of RHY and ISOR were detected in urine and feces. Glucuronic acid conjugates of hydroxylated RHY (M1a and M2a) and hydroxylated ISOR (M1b and M2b) were detected in rat bile. The detection of RHY and ISOR in rat brain shows that these alkaloids could pass through the BBB. However, all of the metabolites of RHY (M1a, M2a, M3a, and M4a) and ISOR (M1b, M2b, M3b, and M4b) were found not to enter the circulation system on the basis of LC-MS analysis.

Kampo formulations usually contain multiple number of medicinal herbs. Due to the complex nature in the constituents of the formulations, detection of bioactive constituents is one of the hard challenges; it is even more difficult to detect the constituents and metabolites in vivo. The present study provided a successful example to use LC-MS to quantify the major bioactive constituents in Kampo formulations and to monitor their concentration changes in vivo. As traditional medicines are usually used as formulations, pharmacokinetics studies on the bioactive constituents after oral administration of a formulation can provide more accurate and important information.

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References

11) Reichen, J. and Le, M.: Influence of common bile duct cannula size on maximal secretory rate of taurocholate in

