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Research Article DRUG METABOLISM AND DISPOSITION

3-*epi*-18*β*-glycyrrhetinic acid or its glucuronide, the metabolites of glycyrrhizinic acid with individual differences, correlated with diagnostic maker for licorice-induced pseudoaldosteronism in humans

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Running title

Individual difference and pseudoaldosteronism by licorice

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ABBREVIATIONS: 11*β*-HSD2, 11*β*-hydroxysteroid dehydrogenase type 2; 18*α*-GA, 18*α*-glycyrrhetinic acid; 22-OH-GA3S, 22*α*-hydroxy-18*β*-glycyrrhetinyl-3-*O*-sulfate; 22-OH-GA3S30G, 22*α*-hydroxy-18*β*-glycyrrhetinyl-3-*O*-sulfate-30-glucuronide; 3-*epi*-GA, 3-*epi*-18*β*-glycyrrhetinic acid; 3-*epi*-GA30G, 3-*epi*-18β-glycyrrhetinyl-30-*O*-glucuronide; 3MGA, 3-monoglucuronyl-18*β*-glycyrrhetinic acid; COSY, correlation spectroscopy; EHBR, Eisai hyperbilirubinemic rats; GA, 18*β*-glycyrrhetinic acid; GA3G, 18*β*-glycyrrhetinyl-3-*O*-glucuronide; GA3S, 18*β*-glycyrrhetinyl-3-*O*-sulfate; GA3S30G, 18*β*-glycyrrhetinyl-3-*O*-sulfate-30-*O*-glucuronide; GA30G, 18*β*-glycyrrhetinyl-30-*O*-glucuronide;

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GL, glycyrrhizinic acid; HMBC, heteronuclear multiple bond correlation; HSQC. heteronuclear single quantum coherence; LC, liquid chromatography; MR, mineralocorticoid receptor; MRM, multiple reaction monitoring; MS, mass spectrometry; PAC, plasma aldosterone concentrations; PsA, pseudoaldosteronism; ROESY, rotating frame overhauser effect spectroscopy.

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ABSTRACT

Licorice is a crude drug that is used in traditional Japanese Kampo medicine and is also used as a sweetener. Occasionally, it causes pseudoaldosteronism (PsA) as a side effect. The major symptoms include hypokalemia, hypertension, edema, and low plasma aldosterone levels. PsA might be caused by the metabolites of glycyrrhizinic acid (GL), a component of licorice. The development of PsA markedly varies among individuals; however, the factors that cause these individual differences remain unknown. In this study, 78 patients who consumed Kampo medicines containing licorice were enrolled, and their laboratory data, including serum potassium levels, plasma aldosterone concentrations (PAC), and the concentrations of GL metabolites in the residual blood and/or urine samples were evaluated. Of the 78 participants, 18*β*-glycyrrhetinic acid (GA), 3-*epi*-GA, 3-oxo-GA, 18*β*-glycyrrhetinyl-30-*O*-glucuronide (GA30G), and 3-*epi*-GA30G were detected in the serum samples of 65, 47, 63, 62, and 3 participants, respectively. Of the 29 urine samples collected, GA30G and 3-*epi*-GA30G were detected in 27 and 19 samples. 3-*epi*-GA30G is a newly found GL metabolite. Moreover, 3-*epi*-GA, 3-oxo-GA, and 3-*epi*-GA30G were identified in human samples for the first time. High individual differences were found in the appearances of 3-*epi*-GA in serum and 3-*epi*-GA30G in urine, and the concentrations of these metabolites were correlated with serum PsA markers. The inhibitory titers of 3-*epi*-GA, 3-oxo-GA, GA30G, and 3-*epi*-GA30G on human 11*β*-hydroxysteroid dehydrogenase type 2 (11*β*-HSD2) were almost similar. These findings suggest that 3-*epi*-GA and/or 3-*epi*-GA30G are associated with individual differences in the development of PsA.

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SIGNIFICANCE STATEMENT

In this study, we detected 3-*epi*-GA in human serum for the first time. We also identified 3-*epi*-GA30G as a novel GL metabolite in human urine. These GL metabolite levels showed correlations with markers of PsA. Additionally, there are individual differences in whether or not they appear in the serum/urine. In conclusion, 3-*epi*-GA/3-*epi*-GA30G correlates with individual differences in the development of PsA.

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Introduction

Licorice is the dried root and stolon of *Glycyrrhiza glabra* or *G. uralensis*, and is used as a crude drug in traditional Japanese Kampo medicine and other modalities of traditional East Asian medicines. Licorice is contained in about 70% of Kampo ethical formulations approved by the Japanese government, mainly to harmonize crude drugs in formulations without targeting specific diseases. Licorice contains glycyrrhizinic acid (GL; Fig. 1), which is used in Western medicine to treat inflammatory diseases and hepatitis (Zhang et al., 2002).

Licorice consumption and GL use are known to cause pseudoaldosteronism (PsA), which is characterized by hypokalemia, hypertension, edema, and low plasma aldosterone concentrations (PAC) (Conn et al., 1968). Therefore, the safety of GL needs to be determined on a global scale (Joint FAO/WHO Expert Committee on Food Additives, 2005). Pseudoaldosteronism (PsA) is hypothesized to be caused by GL metabolites (Ploeger et al., 2001). Normally, aldosterone binds to the mineralocorticoid receptor (MR) that is expressed in the renal tubules to maintain the homeostasis of blood potassium and sodium concentrations. In normal kidneys, cortisol is metabolized to cortisone by 11β-hydroxysteroid dehydrogenase type 2 (11β-HSD2). Cortisone does not affect the MR via its low affinity for this receptor; however, when GL metabolites inhibit 11β-HSD2, the excess cortisol acts on the MR, leading to PsA (Whorwood et al. 1993; Tanahashi et al. 2002). The development of PsA is independent of the dose, duration, or GL content of licorice, and varies widely according to the patient's constitution (Morimoto and Nakajima, 1991). We have reported several patient factors related to licorice-induced PsA including constipation, hypoalbuminemia, hyper direct-bilirubin, older age, and concomitant medications along with daily dosage of licorice and long-term use of it (Yoshino et al. 2021).

Although several GL metabolites have been reported, the factors responsible for individual differences are unknown. Orally administered GL is hydrolyzed by intestinal

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bacteria to 18*β*-glycyrrhetinic acid (GA; Fig. 1), absorbed into circulation (Hattori et al., 1983), and further metabolized in the body. The glucuronyl conjugate of GA at C-3, 18*β*-glycyrrhetinyl-3-*O*-glucuronide (GA3G) (Fig. 1), was found in the blood of patients with licorice-induced PsA (Kato et al.,1995). 18*β*-glycyrrhetinyl-30-*O*-glucuronide (GA30G, **5**; Fig. 1), the glucuronyl conjugate of GA at C-30, has also been detected in the urine of patients that take a Chinese medicinal product containing licorice (Lan et al., 2021).

Previously, we identified 22*α*-hydroxy-18*β*-glycyrrhetinyl-3-*O*-sulfate-30-glucuronide (22-OH-GA3S30G, **1**), 22*α*-hydroxy-18*β*-glycyrrhetinyl-3-*O*-sulfate (22-OH-GA3S, **2**), 18*β*-glycyrrhetinyl-3-*O*-sulfate (GA3S, **3**), and 18*β*-glycyrrhetinyl-3-*O*-sulfate-30-*O*-glucuronide (GA3S30G, **4**) as GL metabolites (Fig. 1) in the urine of Eisai hyperbilirubinemic rats (EHBRs) orally treated with GA (Morinaga et al., 2018; Ishiuchi et al., 2019, 2021). In addition, we detected GA3S and GA3S30G in the serum of patients who consumed licorice (Ishiuchi et al., 2019, 2021).

As GA was not transported into tubular epithelial cells, where 11*β*-HSD2 exists, owing to its high binding ratio to serum albumin and no recognition as the substrate of organic anion transporters expressed on the cells, and as GA3G was not detected in the serum of most patients with PsA, we hypothesized that GA3S and GA3S30G might be the causative agents of PsA (Takahashi et al., 2019; Ishiuchi et al., 2021). However, no clear explanation is available for the individual differences in licorice susceptibility as these metabolites correlated well with the daily licorice dosage.

In our previous study, we found an unknown peak at different retention time in the multiple reaction monitoring (MRM)-mass spectrometry (MS) chromatogram of GA when the serum concentrations of these metabolites were analyzed using liquid chromatography (LC)-MS/MS. As the MS fragment spectrum of this unknown peak was the same as that of GA, this peak must be derived from the stereoisomers of GA.

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3-*epi*-18*β*-glycyrrhetinic acid (3-*epi*-GA, **6**; Fig. 1) is a stereoisomer of GA that was first identified as the metabolite of GL by human intestinal bacteria *in vitro* (Hattori M, et al., 1983). Some intestinal bacteria oxidize the hydroxy group of GA at C-3 to produce 3-oxo-18*β*-glycyrrhetinic acid (3-oxo-GA, **7**; Fig. 1) and reduce 3-oxo-GA to produce 3-*epi*-GA (Hattori M, et al., 1983). Therefore, we predicted that this unknown peak in the MRM-MS chromatogram of GA from human serum could be derived from 3-*epi*-GA.

In this study, we prepared 3-*epi*-GA from GL and identified that the unknown peak represents 3-*epi*-GA based on its retention time on the MRM-MS chromatogram of GA. Furthermore, we prepared 3-oxo-GA from GA and 3-*epi*-GA30G from 3-*epi*-GA, and reanalyzed the concentrations of these GL metabolites in the serum and urine samples of patients enrolled in our previous study (Takahashi et al., 2019). Individual differences were found in the appearance of 3-*epi*-GA in the serum and 3-*epi*-GA30G in the urine of patients and a correlation was identified between the concentrations of 3-*epi*-GA in serum or 3-*epi*-GA30G in urine and serum PsA markers.

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Materials and Methods

Participants. We conducted a multicenter study comprising patients who visited the Center for Kampo Medicine at Keio University Hospital, the Department of Japanese Oriental (Kampo) Medicine at Chiba University Hospital, the Clinic of Japanese Oriental (Kampo) Medicine at Kanazawa University Hospital, and the Department of Oriental Medicine at Kameda Medical Center from November 2011 to July 2018. Most of the participants in the present analysis had been taking Kampo medicinal products containing licorice daily for more than two weeks and most of the participants' last dose was the same morning of the sample collection. Serum and spot urine samples were collected according to the clinical suspicion for PsA, and were pre-treated as described in the protocol reported in our previous studies (Takahashi et al., 2019; Ishiuchi et al., 2021). In May 2021 and March 2023, fresh human feces were collected from a patient who had not consumed licorice for more than one month. All patients were registered at the hospital and provided written informed consent. The study design was approved by the appropriate institutional review boards at Keio University, Chiba University, Kanazawa University, and Kameda Medical Center.

Materials. GA3S and GA3S30G were prepared as described previously (Morinaga et al., 2018; Ishiuchi et al., 2019, 2021). GA was purchased from Fujifilm Wako Pure Chemicals (Osaka, Japan). GA3G and UDP-glucuronic acid were obtained from Nacalai Tesque (Kyoto, Japan). GL ammonium salt and subtilisin were purchased from Sigma-Aldrich (St. Louis, MO, USA). Carbenoxolone and Dess-Martin periodinane were obtained from LKT Laboratories (St. Paul, MN, USA) and Tokyo Chemical Industry (Tokyo, Japan), respectively. Alamethicin and saccharolactone were purchased from Cayman Chemical Company (Ann Arbor, MI, USA) and Toronto Research Chemicals (Toronto, Canada), respectively. Human kidney microsomes and 1,2,6,7-[^{3}H]-cortisol were obtained from Sekisui XenoTech (Kansas City, KS, USA) and PerkinElmer Life and Analytical Sciences (Waltham, MA, USA), respectively. The

donor information of this microsome product shown in the package insert is shown in Table S1.

Animal Experiment. A rat liver microsome fraction (RLM) was prepared from 8-week-old male rats (Japan SLC, Hamamatsu) as previously described (Makino et al. 2006). The animal experimental procedures were approved as #23-011 by the Animal Care Committee at the Graduate School of Pharmaceutical Sciences, Nagoya City University, Nagoya, in accordance with the guidelines of the Japanese Council on Animal Care.

Preparation of the GL metabolites. 3-*epi*-18*β*-glycyrrhetinic acid (3-*epi*-GA) was prepared from GL as previously described (Kobashi et al., 1984). Briefly, 30 mL of Gifu anaerobic medium broth (Nissui Pharmaceutical, Tokyo, Japan) and 6 g of fresh human feces were placed in CO_2-filled anaerobic jars containing Anaero Pack®-Anaero (Mitsubishi Gas Chemical, Tokyo, Japan) and centrifuged at 16 × *g* for 1 min. The supernatant was then used as the intestinal bacterial mixture. GL ammonium salt (250 mg) and the intestinal bacterial mixture (25 mL) were added to 225 mL of Gifu anaerobic medium broth and incubated under the anaerobic conditions at 37 °C for 72 hr with shaking (20 rpm). The pH of the reaction mixture was adjusted to approximately 1 using concentrated HCl and the extraction was carried out with ethyl acetate (EtOAc). The EtOAc layer was concentrated under reduced pressure, subjected to silica gel column chromatography, and eluted with $CHCl_3$/EtOAc/acetic acid (80:20:0.5). Further separation was achieved via HPLC (column, COSMOSIL 5C_{18}-AR-II (Nacalai, Tesque), 5 μm, 10 mm I.D. × 250 mm; mobile phase, acetonitrile/H_2O/trifluoroacetic acid (TFA) (67:33:0.1); flow rate, 2.5 mL/min; detection, UV at 254 nm), which yielded 3-*epi*-GA (4 mg) and 3-oxo-GA (0.1 mg). This process was repeated on a 7.4-fold scale, yielding an additional 41 mg of 3-*epi*-GA.

3-oxo-18*β*-glycyrrhetinic acid (3-oxo-GA) was prepared from GA (50 mg) treated with Dess-Martin periodinane (2 equivalents) in CH_2Cl_2 at room temperature for 1 hr.

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The product was then purified via HPLC (column, COSMOSIL 5C_{18}-AR-II, 5 μm, 20 mm I.D. × 250 mm; mobile phase, acetonitrile/H_2O/TFA (70:30:0.1); flow rate, 8 mL/min; detection; UV at 254 nm), which yielded 3-oxo-GA (15 mg).

18*β*-glycyrrhetinyl-30-*O*-glucuronide (GA30G) was prepared from GA by partly modifying the general glucuronidation reaction method (Fisher et al., 2000). GA (24 mg), alamethicin (50 μg/mL), saccharolactone (5 mM), $MgCl_2$ (1 mM), UDP-glucuronic acid (5 mM), and RLM (2 mg protein/mL) were mixed in 20 mL of phosphate buffer (100 mM, pH 7.1) and incubated at 37 °C for 24 hr. The reaction was stopped by adding twice the volume of methanol (MeOH). The reaction mixture was sequentially extracted with 1-hexane and $CHCl_3$. After adjusting the pH of the water layer to 2 using concentrated HCl, further extraction was performed using EtOAc. The EtOAc layer was concentrated under reduced pressure, separated using silica gel column chromatography, and eluted with $CHCl_3$/MeOH/H_2O/TFA (1:0:0:0 to 6:4:1:0.01, stepwise). The fraction containing GA30G was further purified via HPLC (column, COSMOSIL 5C_{18}-AR-II, 5 μm, 10 mm I.D. × 250 mm; mobile phase, acetonitrile/H_2O/TFA (47:53:0.1); flow rate, 0.6 ml/min; detection, UV at 254 nm), yielding GA30G (0.2 mg) as a sodium salt.

The chemical structures of 3-*epi*-GA, 3-oxo-GA, and GA30G were identified using spectroscopic data, including ^{1}H and ^{13}C NMR, two-dimensional (2D) NMR, and MS spectra shown in Supplements, and the data from previous studies (Hattori et al., 1983, Kanaoka et al., 1986).

3-*epi*-18*β*-glycyrrhetinyl-30-*O*-glucuronide (3-*epi*-GA30G, **8**; Fig. 1) was also prepared from 3-*epi*-GA in a similar manner to the preparation of GA30G. 3-*epi*-GA (21.6 mg), alamethicin (25 μg/mL), saccharolactone (5 mM), $MgCl_2$ (1 mM), UDP-glucuronic acid (5 mM), and RLM (2 mg protein/mL) were mixed in 600 mL of phosphate buffer (100 mM, pH 7.1) and incubated at 37 °C for 24 hr. The EtOAc-soluble materials in the reaction mixture (191 mg) were separated via silica gel

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column chromatography and eluted stepwise using $CHCl_3$/MeOH (1:0 – 1:1). The fraction containing 3-*epi*-GA30G was further purified via HPLC (column, COSMOSIL 5C_{18}-AR-II, 5 μm, 4.6 mm I.D. × 250 mm; mobile phase, acetonitrile/H_2O/TFA (50:50:0.1); flow rate, 0.6 ml/min, detection; UV at 254 nm), and 3-*epi*-GA30G (0.9 mg) was obtained as a sodium salt.

3-*epi*-18*β*-glycyrrhetinyl-30-*O*-glucuronide (sodium salt): $[\alpha]_D^{22}$ +78 (*c* 0.35, MeOH); ^{1}H NMR (CD_3OD, 500 MHz) and ^{13}C NMR (CD_3OD, 125 MHz), see Table 1; ESIMS *m/z* 691 $[M+Na]^+$; HRESIMS *m/z* 691.3436 $[M+Na]^+$ (calcd for $C_{36}H_{53}O_{10}Na_2$, 691.3434).

Quantitation assay for the GL metabolites in human serum and urine. The concentrations of 3-*epi*-GA, 3-oxo-GA, GA30G, and 3-*epi*-GA30G were measured as described below. Serum and urine samples (100 μL) were mixed with 10 μL of subtilisin (0.91 U/mL) and incubated at 37 °C for 30 min to release GL metabolites from albumin. The reaction was terminated using 1 mL of ice-cold EtOH containing carbenoxolone (500 nM) as the internal standard and then held at −20 °C for 30 min. The solutions were centrifuged at 18,000 × *g* for 7 min at 4 °C, and the supernatants were transferred to a new tube, dried at 40 °C, and lyophilized. The residues were dissolved in 100 μL of 1% formic acid and 50% acetonitrile in H_2O and centrifuged at 18,000 × *g* for 10 min at 4 °C. The supernatants (10 μL) were analyzed using LC-MS/MS as follows: System, Quattro Premier XE (Waters Corporation, Milford, MA, USA); column, Capcell Pak ADME (3 μm, 2.1 mm I.D. × 150 mm, Osaka Soda, Osaka, Japan); mobile phase, 0.1% formic acid in H_2O / 0.1% formic acid in acetonitrile (4:6) at 0.25 mL/min; and column temperature, 23 °C. The transitions (precursor-to-product) in electrospray ionization (ESI) (+)-MRM mode were monitored. The retention times of the targets were 647.4 to 471.6 *m/z* for GA30G and 3-*epi*-GA30G (4.2 and 4.8 min, respectively); 471.3 to 91.0 *m/z* for GA and 3-*epi*-GA (12.5 and 14.0 min, respectively); 571.8 to 453.6 *m/z* for carbenoxolone (15.1 min); and 469.3 to 148.9 *m/z* for 3-oxo-GA

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(17.8 min). Each compound was identified by a comparison with the retention time of the sample in the MRM-MS chromatograms. The peak-area ratio of the compounds to their internal standards and the least-squares method ($r^2 > 0.98$) were used to examine a linear regression over the concentration range of 1.0 nM to 2.0 μM for each compound. When the detected concentrations were out of the ranges, the samples were diluted 10-fold with H_2O and reanalyzed to fit the detectable ranges.

Evaluation of the relationship between GL metabolites and PsA markers. Serum potassium levels and PAC were obtained from the laboratory data at each participating institution. Samples were processed and measured at individual institutions according to each institution's protocol.

Determination of *in vitro* 11*β*-HSD2 inhibitory activity using human kidney microsomes. Assays were carried out using human kidney microsomes and 1,2,6,7-[^{3}H]-cortisol as described by Diederich et al. (Diederich et al.,2000) with slight modifications as previously described (Makino et al., 2012). Briefly, [^{3}H] cortisol and each GL metabolite were mixed with human kidney microsome product, and incubated at 37 °C for 30 min. Thereafter, the amount of [^{3}H] cortisone was measured. Half maximal inhibitory concentration (IC_{50}) was calculated from the formula obtained by the method of least squares using three points across 50% in one set of substrate concentrations, and then this was repeated four times to obtain the mean IC_{50} values ± standard error (S.E.) ($n = 4$).

Statistics. The statistical analyses related to Fig. 2, Tables 5 and 8 were performed using Spearman's test on Excel Statistics (version 7, Esumi, Tokyo, Japan).

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Results

Preparation of 3-*epi*-GA30G from 3-*epi*-GA. In the present study, we independently prepared four GL metabolites: 3-*epi*-GA, 3-oxo-GA, GA30G, and 3-*epi*-GA30G. Among them, only 3-*epi*-GA30G was previously unreported. To obtain this compound, 3-*epi*-GA was treated with RLM in a similar manner to GA30G. The EtOAc-soluble materials in the reaction mixture were separated using silica gel column chromatography and C_{18} HPLC to yield compound **8**. The molecular formula of compound **8** was identified as $C_{36}H_{53}O_{10}Na$ based on HRESIMS [*m/z* 691.3436 $(M+Na)^+$, Δ+0.2 mmu]. The ^1H and ^{13}C NMR (Table 1) and heteronuclear single quantum coherence (HSQC) spectra of compound **8** were similar to those of 3-*epi*-GA, except for one signal (δ_C 176.8), and the additional six signals (δ_C 175.6; δ_C 95.5, δ_H 5.54; δ_C 78.1, δ_H 3.46; δ_C 77.3, δ_H 3.72; δ_C 73.9, δ_H 3.38; δ_C 73.4, δ_H 3.50) indicated a new glucuronyl group in the NMR spectra. The planar structure of compound **8** was elucidated by analyzing the 2D NMR data, including the ^1H-^1H correlation spectroscopy (COSY), HSQC, and heteronuclear multiple bond correlation (HMBC) spectra in CD_3OD. The aglycon part of compound **8** was the same as that of glycyrrhetinic acid on the basis of the 2D NMR analyses. Furthermore, the key connection of a β-glucuronyl group to C-30 in compound **8** was revealed by the HMBC correlation for H-1' (δ_H 5.54) to C-30 (δ_C 176.8). The relative stereochemistry of compound **8** was verified as the desired structure based on the rotating frame overhauser effect spectroscopy (ROESY) spectrum. Thus, prepared compound **8** was confirmed to be confirmed to be a sodium salt of 3-*epi*-18β-glycyrrhetinyl-30-*O*-glucuronide.

GL metabolites in the participants. Overall, 78 patients were enrolled in this study and 88% were female. The demographic data and clinical background of patients are shown in Tables 2 and 3. There were no differences in patient backgrounds among the sites, and the differences in reference values were negligible, but missing values were concentrated in one facility.

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The concentrations of GL metabolites in the serum and urine samples were measured using LC-MS/MS and are shown in Table 4. Of the 78 participants, GA, 3-oxo-GA, and GA30G were detected in 83%, 81%, and 79%, respectively, of the serum samples collected; however, 3-*epi*-GA was only detected in 60% of the samples. Of note, the maximum serum concentrations of GA and 3-*epi*-GA were similar (1.2 μM for GA and 1.9 μM for 3-*epi*-GA). Among the 29 urine samples obtained, GA30G was detected in 93% of the samples, while 3-*epi*-GA30G was only detected in 66% of the samples. The maximum urinary concentration of GA30G was approximately 7.5-fold higher than that of 3-*epi*-GA30G. The appearance of 3-*epi*-GA in the serum and that of 3-*epi*-GA30G in the urine had larger individual differences than those of the other GL metabolites.

Positive correlations were observed between the serum concentration of 3-*epi*-GA and daily licorice dosage; serum concentrations of GA and 3-*epi*-GA; and serum concentrations of 3-*epi*-GA and 3-oxo-GA (Fig. 2). The correlation coefficient (*r*-value) between the serum concentrations of 3-*epi*-GA and 3-oxo-GA was larger than that between the serum concentrations of 3-*epi*-GA or GA and daily dosage of licorice. 3-*epi*-GA could not be detected in several samples with relatively high GA concentrations or samples from participants taking relatively high daily dosages of licorice.

The urinary concentration of GA30G had a positive correlation with the serum concentrations of GA, 3-*epi*-GA, 3-oxo-GA, and GA30G, respectively (Table 5, Supplementary Fig. S1). Urinary 3-*epi*-GA30G concentration was and positively correlated with the serum concentrations of GA, 3-*epi*-GA, and 3-oxo-GA, respectively, but not with that of GA30G.

Serum concentrations of the GL metabolites in a patient with PsA. In our previous studies (Ishiuchi et al, 2019; Takahashi et al, 2019), we evaluated the serum concentration profile of GL metabolites in a 76-year-old woman prescribed Kampo

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medicinal products for 3 years (1.5 g licorice for 1 year and 2 months and 3 g for 1 year and 10 months) and developed PsA. On day 0, the patient stopped taking the Kampo products, and blood samples were collected on days 0, 5, 9, 11, and 15. In the present study, the concentrations of GA3S30G, 3-*epi*-GA, 3-oxo-GA, GA30G, and 3-*epi*-GA30G were measured in the same samples. Serum sample on Day 5 was completely depleted in the previous analyses. The concentration of 3-*epi*-GA on day 0 was higher than that of GA, and the elimination of 3-*epi*-GA and 3-oxo-GA tended to be slower than that of GA (Table 6).

Associations between the serum and urine concentrations of GL metabolites and clinical data. Of the 78 patients, 31 in whom 3-*epi*-GA was not detected in their serum did not exhibit hypokalemia. However, of the 47 patients with 3-*epi*-GA detected in their serum, seven developed hypokalemia and ultimately diagnosed as PsA, and one developed a PAC level below the detection limit (< 36 pg/dL). There were no statistical differences between these seven patient group and others regarding disease conditions and clinical background, such as the symptom of PsA, concomitant medications, sex, and age. Plasma aldosterone concentration (PAC) was tended to be less in the group with 3-*epi*-GA compared to those of absence (Table 7). The serum concentration of 3-*epi*-GA was negatively correlated with serum potassium level and PAC. In addition, 3-oxo-GA in serum and 3-*epi*-GA30G in urine had a negative correlation with these PsA markers. However, GA30G in serum and urine did not correlate with PsA markers (Table 8, Supplementary Fig. S2). No association was found between increased blood pressure or edema and the blood or urine concentrations of GL metabolites.

Inhibitory effects of 3-*epi*-GA, 3-oxo-GA, GA30G, and 3-*epi*-GA30G on human 11*β*-HSD2. GA inhibited human 11*β*-HSD2 in a concentration-dependent manner, with IC_{50} value of 0.50 ± 0.10 μM. 3-*epi*-GA, 3-oxo-GA, GA30G, and 3-*epi*-GA30G also inhibited human 11*β*-HSD2 in concentration-dependent manners. The IC_{50} values of these metabolites were 0.17 ± 0.02, 0.44 ± 0.25, 0.87 ± 0.16, and 0.48 ± 0.08 μM,

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respectively (Fig. 3).

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Discussion

In this study, we identified four GL metabolites, 3-*epi*-GA, 3-oxo-GA, GA30G, and 3-*epi*-GA30G, in the serum or urine of patients who had consumed licorice. Previously, 3-*epi*-GA and 3-oxo-GA were identified as the metabolites of GL in a human intestinal bacterial mixture *in vitro* (Hattori et al., 1983). However, these investigators did not detect 3-*epi*-GA and 3-oxo-GA in the blood of patients administered GL. Therefore, to our knowledge, the present study is the first to demonstrate the presence of these GL metabolites in the serum of patients. Furthermore, we identified 3-*epi*-GA30G as a novel GL metabolite found in the urine of patients for the first time.

Seventy-eight patients were enrolled in this study, and 88% were female. In Japan, females prefer to use Kampo medicines compared with males, and previously we have reported that 85% of Japanese PsA patients who developed rhabdomyolysis were female (Yoshino et al, 2014). Individual differences were observed in the appearance of 3-*epi*-GA compared to GA in the serum of patients. The detection ratios (number of detections over the number of specimens measured) of 3-*epi*-GA and GA in the serum were 60% and 83%, respectively. Hattori et al. (Hattori et al., 1985) and Akao et al. (Akao et al., 1990) found that *Peptostreptococcus intermedius*, *Clostridium perfringens*, *Eubacterium* sp., and *Ruminococcus* sp. hydrolyzed GL to GA; *Ruminococcus* sp. and *C. innocuum* oxidized GA to 3-oxo-GA; *Ruminococcus* sp. reduced 3-oxo-GA to GA; and *C. innocuum* reduced 3-oxo-GA to 3-*epi*-GA. These results suggest that patients in whom 3-*epi*-GA was not detected might not have bacterial species, such as *C. innocuum* to reduce 3-oxo-GA to 3-*epi*-GA, and individual differences in the appearance of 3-*epi*-GA in the serum would depend on the presence of these bacterial species in intestinal bacterial flora.

Lan et al. (Lan et al., 2021) exhaustively analyzed GL metabolites in the serum of 14 Chinese patients who consumed medicinal products containing licorice; however, they did not find 3-*epi*-GA in any of the patients. This result suggests that the

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differences in race or dietary habits between Japanese and Chinese people might affect the appearance of 3-*epi*-GA in the serum of individuals consuming licorice possibly due to the difference in gut microbiota, or it was possible that Lan et al. missed 3-*epi*-GA. As 3-*epi*-GA was not detected in rat samples in our previous studies (Makino et al., 2008, 2012), we assumed that the pharmacokinetics and bioavailability of GL metabolites and the appearance of 3-*epi*-GA in serum were affected by the activity of specific enterobacteria among patients and experimental animals.

In the present study, another GL metabolite, GA30G, was detected in 79% of serum samples and 93% of urine samples. Of note, GA30G was first identified as a GL metabolite in rat bile (Iveson et al., 1971). By derivatizing GA3G and GA30G from GA, Kanaoka et al. (Kanaoka et al., 1986) found GA3G instead of GA30G in the serum of a female patient daily injected with GL. GA is conjugated with glucuronic acid by uridine 5'-diphospho-glucuronosyltransferases (UGT) 1A1, 1A3, 2B4, and 2B7 (Lu et al., 2009), and GA3G and GA30G are the possible conjugates. Since GA3G was only detected in the serum of 3.1% of the participants at less than 16 nM and was never detected in urine samples, and since Lan et al. (Lan et al., 2021) detected not GA3G but GA30G in the urine of all patients, we propose that GA30G instead of GA3G is the main GA-glucuronyl conjugate in humans. Once GA in circulation is conjugated with glucuronic acid at C-30, this metabolite would be eliminated not only into bile to show an enterohepatic cycle (Ploeger et al., 2000) but also into urine, because GA30G was detected in the urine of 93% of the available urine samples.

Based on the above findings, we predicted that 3-*epi*-GA would also be conjugated with glucuronic acid at C-30 and eliminated into urine. 3-*epi*-GA30G was detected in 66% of the urine samples but was only detected in 3.8% of the serum samples. 3-*epi*-GA30G is a new GL metabolite that was first discovered in human samples. The serum concentration of 3-*epi*-GA30G might be lower than the detectable limit in the present analytical system as the serum concentration of GA30G was approximately

50-fold lower than that in urine and the urine concentration of 3-*epi*-GA30G was markedly lower than that of GA30G. Smaller individual differences in the metabolism of GA or 3-*epi*-GA with glucuronyl conjugation at C-30 and urinary elimination of these conjugates are expected owing to the relatively strong correlations between serum GA and urinary GA30G concentrations and between serum 3-*epi*-GA and urinary 3-*epi*-GA30G.

The serum concentration of 3-*epi*-GA positively correlated with the intake of licorice, the serum concentrations of GA, and 3-oxo-GA, respectively. In our previous study, a correlation was observed between the serum concentration of GA and licorice intake (Takahashi et al., 2019); however, the correlation between the serum concentration of 3-*epi*-GA and licorice intake was weaker than that of GA. This result might be due to individual differences in the appearance of 3-*epi*-GA in the serum, which might be caused by the individual differences in intestinal bacteria owing to the epimerizing process.

In the patient diagnosed as PsA, 3-*epi*-GA and 3-oxo-GA were detected from the end of licorice intake (day 0) to days 11 and 15 post-termination of the intake, respectively. The concentration of 3-*epi*-GA on day 0 was higher than that of GA, suggesting that not only GA3S but also 3-*epi*-GA may relate to the onset of PsA. The serum concentration of GA on day 11 was below the detection limit (Ishiuchi et al, 2019); however, the serum concentration of 3-*epi*-GA on day 11 and that of 3-oxo-GA on day 15 were still above this limit. Therefore, 3-*epi*-GA and 3-oxo-GA may have longer half-lives than GA. According to our previous studies (Ishiuchi et al., 2019, 2021) and the present study, the main metabolic pathways of GA involve sulfonyl conjugation at C-3 and glucuronyl conjugation at C-30. As 3-oxo-GA could not be conjugated at C-3 and could only be conjugated with glucuronic acid at C-30, a longer half-life could be predicted for 3-oxo-GA than GA.

In the present study, 3-*epi*-GA was discovered in human serum owing to an

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unknown peak in the MRM-MS-chromatogram for GA (*m/z* 471.3 to 91.0) (Takahashi et al., 2019). As the peak derived from 3-*epi*-18*β*-glycyrrhetyl-3-*O*-sulfate (3-*epi*-GA3S) was not detected in the MS chromatogram of GA3S (*m/z* 549.5 to 96.5) in the previous study (Takahashi et al., 2019), 3-*epi*-GA may be less conjugated with sulfuric acid than GA at C-3. In our previous study, GA was found to be conjugated with sulfuric acid at C-3 by human sulfotransferase (hSULT) 2A1 (Takahashi et al., 2019), suggesting that the affinity of 3-*epi*-GA for hSULT2A1 may be lower than that of GA and that 3-oxo-GA and 3-*epi*-GA may accumulate in the body more readily than GA. As 3-*epi*-GA, 3-oxo-GA, GA30G, and 3-*epi*-GA30G had similar 11*β*-HSD2 inhibitory activities to GA, 3-*epi*-GA, 3-oxo-GA, and 3-*epi*-GA30G might have a greater contribution to the onset of PsA than GA via their accumulation in the body.

Since the binding rate of GA and to serum albumin is more than 99% (Makino et al., 2012) and GA is not recognized by organic anion transporters 1 and 3 (OAT1/3) expressed in tubular cells, it is predicted that GA and 3-*epi*-GA are difficult to transfer from the circulation into the tubular cells. On the other hand, GA3S, GA3G, and GA3S30G are recognized by OAT1/3 (Makino et al., 2008; Makino et al., 2012; Ishiuchi et al, 2021). Although we did not verify these properties of 3-*epi*-GA, 3-oxo-GA, GA30G, and 3-*epi*-GA30G, the results of previous studies suggest that GA30G and 3-*epi*-GA30G are expected to be recognized by OAT1/3 whereas 3-*epi*-GA and 3-oxo-GA are not.

Individual differences in the appearance of 3-*epi*-GA in serum might be involved in the onset of PsA, which also has high individual differences. All patients without serum 3-*epi*-GA did not exhibit hypokalemia, whereas those developed hypokalemia had serum 3-*epi*-GA. In addition, patients in whom 3-*epi*-GA was detected tended to have lower PAC levels than those in whom this metabolite was not detected. 3-*epi*-GA had a negative correlation with serum PsA markers, suggesting that 3-*epi*-GA may be associated with PsA development. A negative correlation was also found between

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urinary 3-*epi*-GA30G concentration and serum PsA markers.

Previously, we proposed GA3S as a novel PsA marker (Ishiuchi et al, 2019, 2021; Takahashi et al, 2019) owing to the correlation between serum GA3S concentration and serum PsA markers. However, it was detected in almost all patients with or without PsA, making it difficult to consider background factors for individual differences in PsA development. On the other hand, 3-*epi*-GA was detected in all patients with hypokalemia, a symptom of PsA, suggesting that the metabolic process to 3-*epi*-GA, i.e., differences in intestinal bacteria, may be a factor in individual differences in PsA. In addition, as 3-*epi*-GA30G, a glucuronide conjugate of 3-*epi*-GA, can be detected in urine, which can be more easily obtained from patients taking licorice than serum, urinary 3-*epi*-GA30G might be used as a marker to diagnose individual differences in the future onset of PsA and prevent the adverse effects of licorice. In our subsequent study, we will produce a monoclonal antibody to detect 3-*epi*-GA30G with high selectivity and develop an ELISA kit to aid the prevention of PsA onset.

This study had several limitations. Due to the observational nature of this study, we used a single residual serum and spot urine sample from each patient at a general clinical practice in Kampo medicine with different doses of licorice. To reveal the pharmacokinetics of GL and its metabolites, the sequential collection of serum and urine samples over time is required. We did not analyze the active uptake of 3-*epi*-GA30G into tubular cells, and further study is demanded. When assessing the association between GL metabolites and PsA markers, we only identified drugs that may affect K concentrations, such as diuretics, angiotensin receptor blockers or angiotensin-converting enzyme inhibitors, insulin, and glucocorticoids. In addition, it was noted that missing values were concentrated in one facility. Urine samples were obtained from some participants instead of all participants, which may have led to insufficient analysis.

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Author Contributions

Participated in research design: Makino

Conducted experiments: Sakoda, Ishiuchi, Tsunoo

Contributed new reagents or analytic tools: Yoshino, Namiki, Ochiai, Minamizawa, Fukunaga, Watanabe

Performed data analysis: Sakoda, Yoshino

Wrote or contributed to the writing of the manuscript: Sakoda, Ishiuchi, Yoshino, Makino

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Footnotes

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Conflict of Interest Statement

The authors of this manuscript declare the following conflicts of interest: TM and KF received grant support from TSUMURA & CO. TM also received grant support from Kracie Pharmaceuticals, JPS Pharmaceuticals, Kobayashi Pharmaceuticals, and Taisho Holding. TY is employed at Keio University for collaborative research with TSUMURA & CO. KW received a lecture fee from TSUMURA & CO.

Ethics Approval Statement

All procedures performed in the studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and the 1964 Helsinki Declaration and its later amendments or comparable ethical standards.

Patient Consent Statement

All registered participants provided written informed consent, and the study design was approved by the appropriate institutional review boards at Keio University, Chiba University, Kanazawa University, and Kameda Medical Center.

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Data Availability

The data that support the findings of this study are available from the corresponding author, TM, upon reasonable request.

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Figure legends

Fig. 1. Chemical structures of glycyrrhizinic acid (GL) and its metabolites. GA, 18*β*-glycyrrhetinic acid; GA3G, 18*β*- glycyrrhetinyl-3-*O*-glucuronide (called as 3MGA in the previous articles); 22-OH-GA3S30G (**1**), 22*α*-hydroxy-18*β*-glycyrrhetinyl-3-*O*-sulfate-30-glucuronide; 22-OH-GA3S (**2**), 22*α*-hydroxy-18*β*-glycyrrhetinyl-3-*O*-sulfate; GA3S (**3**), 18*β*-glycyrrhetinyl-3-*O*-sulfate; GA3S30G (**4**), 18*β*-glycyrrhetinyl-3-*O*-sulfate-30-*O*-glucuronide; GA30G (**5**), 18*β*-glycyrrhetinyl-30-*O*-glucuronide; 3-*epi*-GA (**6**), 3-*epi*-18*β*-glycyrrhetinic acid; 3-oxo-GA (**7**), 3-oxo-18*β*-glycyrrhetinic acid; 3-*epi*-GA30G (**8**), 3-*epi*-18*β*-glycyrrhetinyl-30-*O*-glucuronide.

Fig. 2. Relationships between serum concentration of 3-*epi*-18*β*-glycyrrhetinic acid (3-*epi*-GA) and the dosage of licorice (a), serum concentration of 18*β*-glycyrrhetinic acid (b), and serum concentration of 3-oxo-GA (c). Each dot represents one case, and the statistical significances of the correlations were evaluated using Spearman's test. The correlation coefficient *r* and *p* values are shown in the graphs. 3-*epi*-GA could not be detected in several samples from participants taking relatively higher daily dosages of licorice.

Fig. 3. Inhibitory effects of 3-*epi*-18*β*-glycyrrhetinic acid (3-*epi*-GA), 3-oxo-GA, glycyrrhetinyl-30-*O*-glucuronide (GA30G), and 3-*epi*-GA30G on type-2 11*β*-hydroxysteroid dehydrogenase (11*β*-HSD2) using human kidney microsome. 18*β*-glycyrrhetinic acid (GA) was used as the positive control. [^{3}H] cortisol and or each glycyrrhizinic acid metabolite were mixed with human kidney microsome fractions, and incubated at 37 °C for 30 min. Thereafter, the amount of [^{3}H] cortisone was measured. Each point is expressed as means ± standard error (S.E: n = 4) of the percentage of [^{3}H]

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cortisone in mixtures without samples.

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TABLE 1

^{1}H and ^{13}C NMR data (CD_3OD) of 3-*epi*-18*β*-glycyrrhetinyl-30-*O*-glucuronide (3-*epi*-GA30G).

Position	δ_H[a]	δ_C[b]	HMBC
1a	2.43 (1H, brd 13.5 Hz)	34.5	3, 9, 25
1b	1.38 (1H, nd[c])		
2a	2.01 (1H, nd[c])	26.2	1a
2b	1.48 (1H, nd[c])		
3	3.33 (1H, nd[c])	76.6	1a, 23, 24
4		38.5	5, 23, 24
5	1.30 (1H, nd[c])	49.3	1a, 3, 7b, 23, 24, 25
6a	1.51 (1H, nd[c])	18.4	5
6b	1.45 (1H, nd[c])		
7a	1.78 (1H, nd[c])	33.8	5, 26
7b	1.43 (1H, nd[c])		
8		46.9	6a, 7a, 9, 15a, 26, 27
9	2.59 (1H, s)	63	5, 7b, 12, 25, 26
10		38.3	1a, 5, 6a, 9, 25
11		202.9	9, 12
12	5.62 (1H, s)	129.1	18
13		172.8	12, 15b, 18, 19b, 27
14		44.7	12, 15a, 16b, 18, 26, 27
15a	1.88 (1H, nd[c])	27.5	16a, 27
15b	1.24 (1H, nd[c])		
16a	2.15 (1H, ddd 13.5, 13.5, 4.0 Hz)	27.5	15a, 18, 28
16b	1.03 (1H, brd 13.5 Hz)		
17		32.9	16a, 18, 19a, 19b, 21a, 22b, 28
18	2.30 (1H, dd 14.0, 4.0 Hz)	49.2	12, 16b, 19a, 19b, 22b, 28
19a	1.90 (1H, nd[c])	42.3	18, 21a, 29
19b	1.73 (1H, dd 13.5, 13.5 Hz)		
20		45.2	19a, 19b, 21a, 22b, 29
21a	2.06 (1H, nd[c])	31.9	19a, 19b, 29
21b	1.45 (1H, nd[c])		
22a	1.49 (1H, nd[c])	38.7	21b, 28
22b	1.38 (1H, nd[c])		
23	0.94 (3H, s)	29.2	5, 24
24	0.87 (3H, s)	22.9	3, 5, 23
25	1.15 (3H, s)	16.9	1b, 5, 9
26	1.14 (3H, s)	19.3	7a, 7b, 9
27	1.45 (3H, s)	23.9	15a, 15b
28	0.84 (3H, s)	28.9	16a, 18, 22a
29	1.22 (3H, s)	28.3	19b
30		176.8	19b, 21b, 29, 1'
1'	5.54 (1H, d 8.0 Hz)	95.5	2', 5'
2'	3.38 (1H, dd 9.0, 8.0 Hz)	73.9	3'
3'	3.46 (1H, dd 9.0, 9.0 Hz)	78.1	1', 2', 4', 5'
4'	3.50 (1H, dd 9.0, 9.0 Hz)	73.4	3', 5'
5'	3.72 (1H, d 9.0 Hz)	77.3	1'
6'		175.6	4', 5'

[a]500MHz. [b]125MHz. [c]nd: *J*-values were not determined because of overlapping with other signals.

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TABLE 2

Demographic data and clinical background of the participants

Item	*n* = 78
Age, year	
Mean ± SD	60 ± 15
Median (min-max)	63 (22 – 87)
Sex, *n* (%)	
Female	69 (88%)
Daily licorice dosage (g)[1)]	
Mean ± SD	2.1 ± 15
Median (min-max)	1.9 (0.5 – 6.0)
Body mass index (kg/m^2)	
Mean ± SD	22 ± 3.6
Median (min-max)	21 (15 – 31)
No information	30
Complication	22 (19 cases)
Liver disease	2
Kidney dysfunction	1
Chronic heart failure	3
Malignancy	16

1) The dosage of licorice in Kampo medicinal products daily taken by patients are shown.

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TABLE 3

Concomitant medications taken by patients

Medications	
K sparing diuretics	9
Thiazides	2
Loop diuretics	3
Other diuretics	2
Ca channel blockers	5
ARB/ACEI	7
α/β blockers	3
Direct renin blockers	0
Glucocorticoids	4
Insulins	0
Thyroid replacements	2
Catecholamines	0
Other unspecified medications	15

ARB, angiotensin receptor blockers.

ACEI, angiotensin-converting enzyme inhibitors.

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TABLE 4

Concentrations of glycyrrhizinic acid (GL) metabolites in serum and urine

	Serum		Urine	
	Concentration	Ratio of detected	Concentration	Ratio of detected
GA	0 – 1.2 μM	65/78	0 – 0.30 μM	11/29
(Median)	(0.094 μM)		(0 μM)	
3-*epi*-GA	0 – 1.9 μM	47/78	N.D.	
(Median)	(0.0082 μM)			
3-oxo-GA	0 – 0.24 μM	63/78	N.D.	
(Median)	(0.0031 μM)			
GA30G	0 – 0.048 μM	62/78	0 – 3.06 μM	27/29
(Median)	(0.0045 μM)		(0.21 μM)	
3-*epi*-GA30G	Trace, 0.0011, 0.0041 μM	3/78	0 – 0.41 μM	19/29
(Median)	(0.0020 μM)		(0.0069 μM)	

Data are expressed as the range of the detected concentrations and their medians. Ratio of detection indicates the number of detections / the number of specimens measured. The concentration "0" indicates less than 0.2 nM. "Trace" indicates 0.2 – 1.0 nM. "N.D." indicates not detected (less than 0.2 nM). "Detected" indicates more than 0.2 nM.

GA, 18*β*-glycyrrhetinic acid, GA30G, 18*β*-glycyrrhetinyl-30-*O*-glucuronide.

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TABLE 5

Relationship between the serum and urine concentrations of glycyrrhizinic acid (GL) metabolites

		vs urine GA30G		*vs* urine 3-*epi*-GA30G	
		Correlation coefficient	*n*	Correlation coefficient	*n*
Serum	GA	0.71***	29	0.58**	19
	3-*epi*-GA	0.60***	29	0.73***	19
	3-oxo-GA	0.62***	29	0.81***	19
	GA30G	0.58***	29	0.29	19
Urine	3-*epi*-GA30G	0.78***	19		

GA, 18*β*-glycyrrhetinic acid, GA30G, 18*β*-glycyrrhetinyl-30-*O*-glucuronide. **$p < 0.01$ and ***$p < 0.001$ exhibit significant correlations evaluated by Spearman's test.

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TABLE 6

Glycyrrhizinic acid (GL) metabolite concentrations in serum of patient with pseudoaldosteronism

	K	GA[1)]	GA3S[1)]	GA3S30G	3-*epi*-GA	3-oxo-GA	GA30G	3-*epi*-GA30G
	(mEq/L)	(nM)	(nM)	(nM)	(nM)	(nM)	(nM)	(nM)
Day 0	2.1	1298	8646	815	1852	701	66	N.D.
Day 5	2.1	647	3623	–	–	–	–	–
Day 9	2.8	11	61	N.D.	4.3	1.8	N.D.	N.D.
Day 11	3.4	N.D.	57	N.D.	2.6	1.5	N.D.	N.D.
Day 15	4.9	N.D.	N.D.	N.D.	N.D.	1.2	N.D.	N.D.

K, potassium levels in serum (marker for PsA); GA, 18*β*-glycyrrhetinic acid; GA3S, 18*β*-glycyrrhetinyl-3-*O*-sulfate; GA3S30G, 18*β*-glycyrrhetyl-3-*O*-sulfate-30-*O*-glucuronide; GA30G, 18*β*-glycyrrhetinyl-30-*O*-glucuronide; N.D., not detected (less than 0.2 nM). Blood samples were collected from patients with PsA on days 0, 5, 9, 11, and 15 after the end of licorice consumption, and serum concentrations of GL metabolites were measured by LC-MS/MS. "–" on day 5 indicates that measurement could not be performed because the sample was completely depleted in the previous analyses. [1)]The concentrations of GA and GA3S were already reported in our previous study (Ishiuchi et al., 2019).

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TABLE 7

Relationship between the detection of 3-*epi*-18*β*-glycyrrhetinic acid (3-*epi*-GA) in blood and pseudoaldosteronism (PsA) markers

	Serum 3-*epi*-GA	
	Detected	Not detected
Total number of cases	47 cases	31 cases
Serum K level (mmol/L)	4.0 (2.6 – 5.1)	4.2 (3.6 – 5.1)
Hypokalemia (< 3.6 mmol/L)	7 cases	0 cases
PAC (pg/dL)	66.4 (10.0 – 235)	133 (51.0 – 259)
PAC below the limit（< 36 pg/dL）	1 case	0 cases

PAC, plasma aldosterone concentration. "Detected" indicates more than 0.2 nM. Data are expressed as the range of the detected concentrations and their medians.

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TABLE 8

Relationships between each metabolite of glycyrrhizinic acid (GL) and pseudoaldosteronism (PsA) markers.

		Serum potassium level		PAC	
		Correlation coefficient	*n*	Correlation coefficient	*n*
Serum	3-*epi*-GA	–0.35**	77	-0.53**	29
	3-oxo-GA	–0.38***	77	–0.50**	29
	GA30G	-0.12	77	–0.36	29
Urine	GA30G	–0.29	29	–0.46*	20
	3-*epi*-GA30G	–0.47*	29	–0.52*	21

PAC, plasma aldosterone concentration; GA, 18*β*-glycyrrhetinic acid; GA30G, 18*β*-glycyrrhetinyl-30-*O*-glucuronide. *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$ exhibit significant correlations based on Spearman's test. One case of serum potassium level and 49 cases of PAC information could not be obtained.

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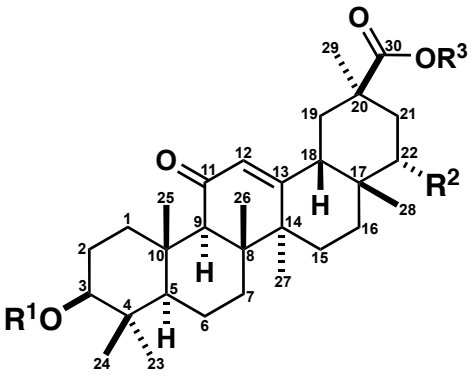

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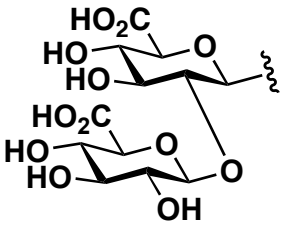

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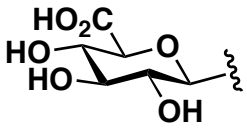
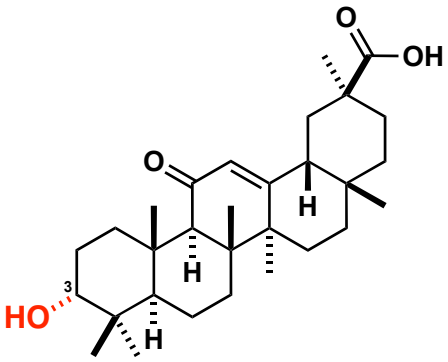
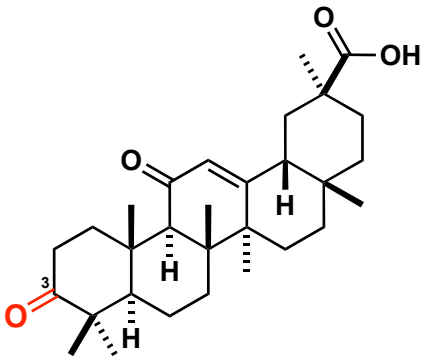


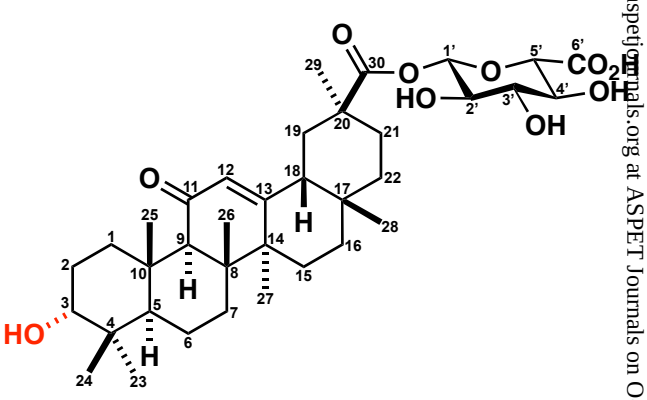
	R^1	R^2	R^3
GL	-glcA-glcA	H	H
GA3G (3MGA)	-glcA	H	H
GA	H	H	H
22OH-GA3S30G (1)	$-SO_3H$	-OH	-glcA
22OH-GA3S (2)	$-SO_3H$	-OH	H
GA3S (3)	$-SO_3H$	H	H
GA3S30G (4)	$-SO_3H$	H	-glcA
GA30G (5)	H	H	-glcA



3-*epi*-GA (6)



3-oxo-GA (7)



3-*epi*-GA30G (8)

Fig. 1

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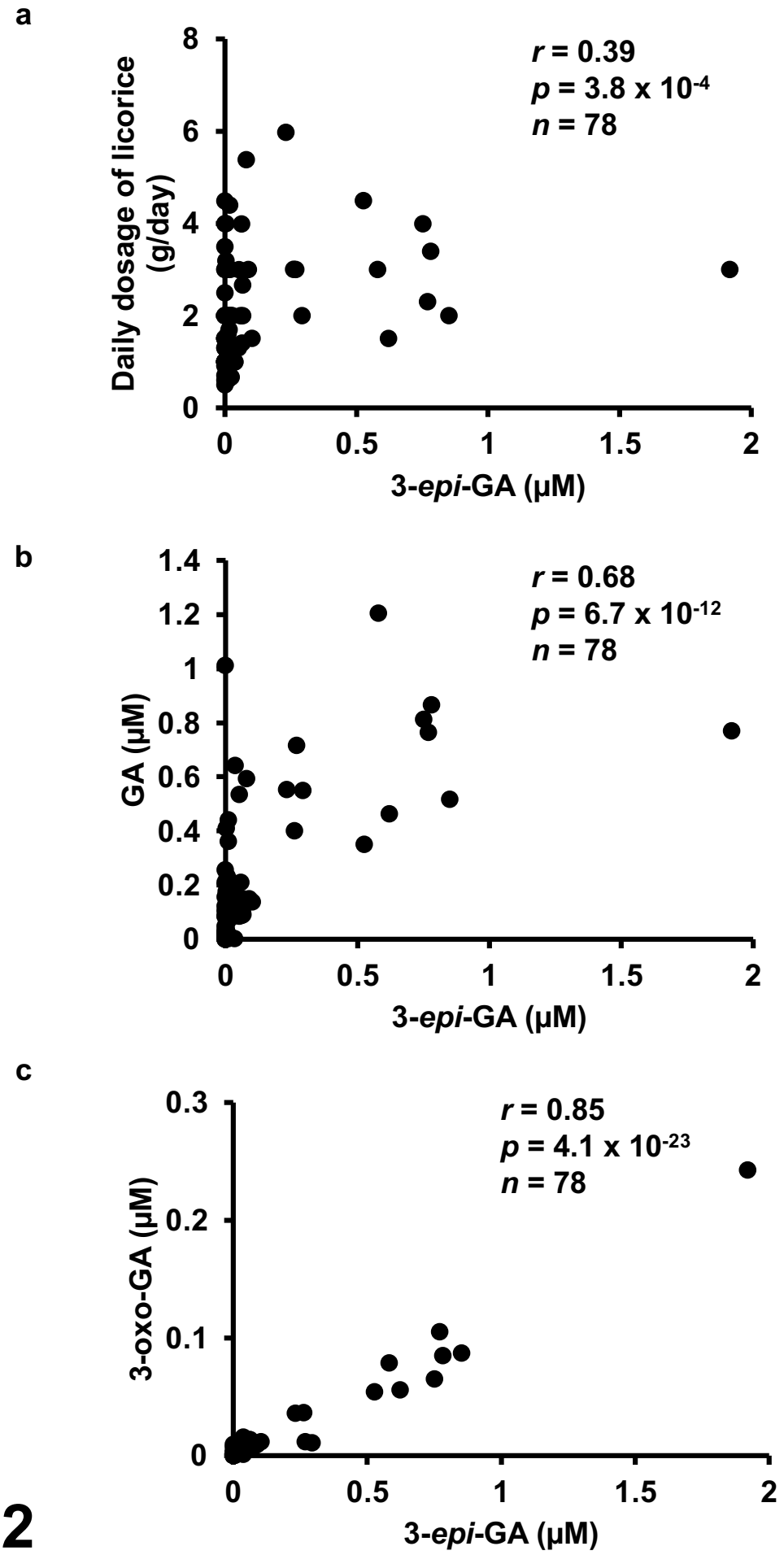

Fig. 2

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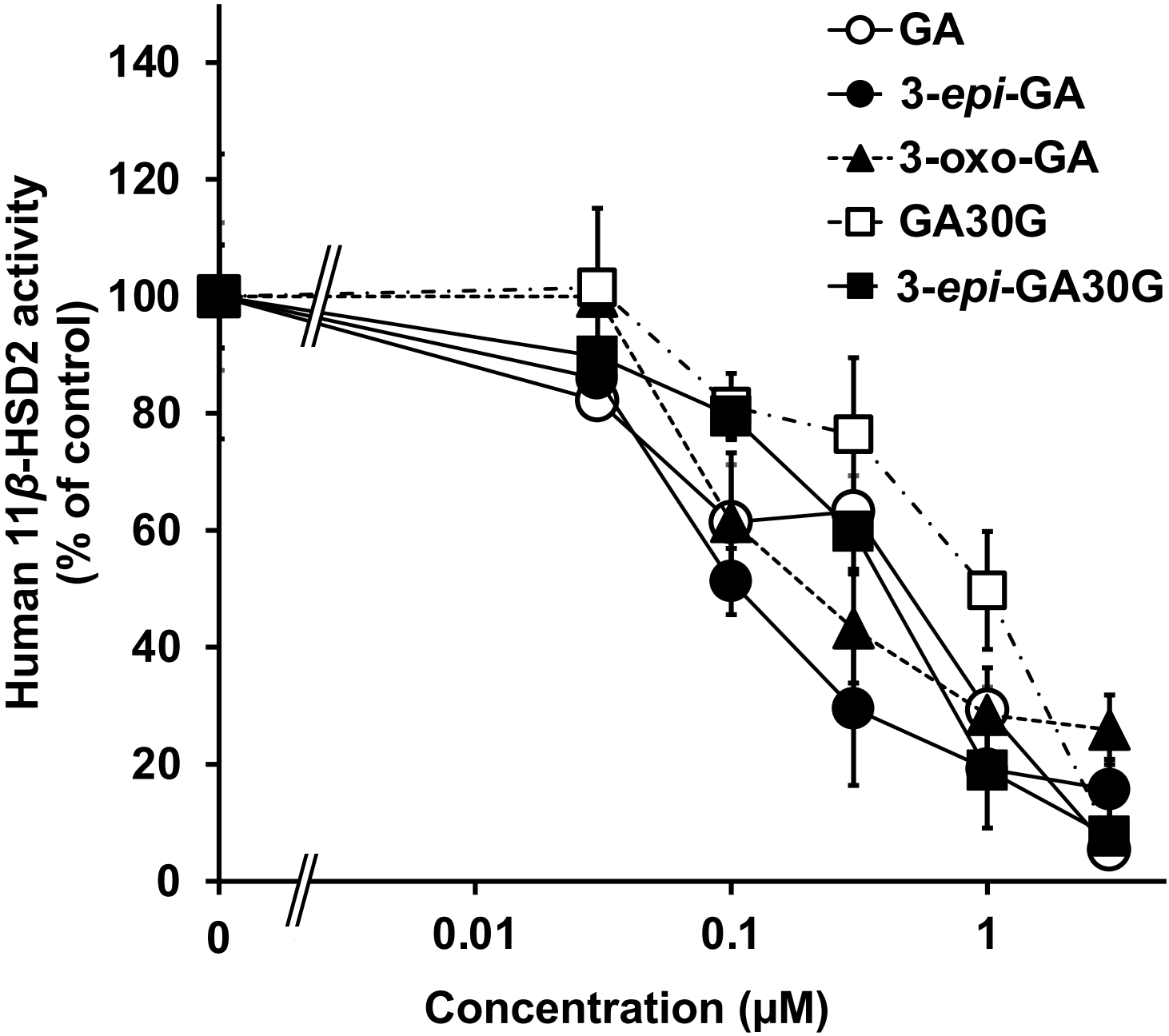

Fig. 3