Study on the bioavailability of prenylated isoflavones, glyceollins, in rats

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Abbreviations

- ABC, ATP-binding cassette
- ACN, acetonitrile
- ADME, absorption, distribution, metabolism, and excretion
- *AUC*, area under the curve
- BCRP, breast cancer-resistant protein
- C3G, cyanidin-3-*O*-glycoside
- CMC, carboxymethyl cellulose
- C_{max}, maximum concentration
- DMSO, dimethyl sulfoxide
- ECG, epigallocatechin-3-*O*-gallate
- EDTA, ethylenediaminetetraacetic acid
- EGCG, (-)-epigallocatechin-3-*O*-gallate
- EIC, extracted-ionization chromatogram
- ER, estrogen receptor
- ESI, electrospray-ionization
- EtOH, ethanol
- E2, 17β-estradiol
- 8PQ, 8-prenyl quercetin
- 8PN, 8-prenyl naringenin
- FA, formic acid
- GlcA, glucuronic acid
- GLP-1, glucagon-like peptide-1
- GLUT4, glucose transporter 4
- HIF, hypoxia-inducible factor
- HMGB1, high mobility group proteins B1
- IVAS, isovanillic acid 3-*O*-sulfate
- IS, internal standard
- ITO, indium-tin oxide
- KBR solution, Krebs-Bicarbonate Ringer's solution
- LC-TOF/MS, liquid chromatography-time-of-flight/ mass spectrometry
- LPS, lipopolysaccharide
- LOD, limit of detection
- MALDI-MS, matrix-assisted laser desorption/ionization mass spectrometry
- MCT, monocarboxylic acid transporter
- Me, methylated
- MeOH, methanol
- MRP, multidrug resistance protein
- NMR, nuclear magnetic resonance
- Nrf2, nuclear factor-erythroid 2
- OAT, organic anion transporter
- OATP, organic anion transporting polypeptides
- 1,5-DAN, 1,5-diaminonaphthalene
- PMSF, phenylmethanesulfonyl fluoride
- PgR, progesterone receptor
- PTs, prenyltransferases

- ROS, reactive oxygen species
- S.D., standard deviation
- *S/N*, signal-to-noise ratio
- SULT, sulfotransferase
- Sul, sulfated
- TF3'G, theaflavin-3'-*O*-gallate
- T_{max}, time of maximum concentration
- $T_{1/2}$, elimination half-life
- UGT, UDP-glucuronosyltransferase

Chapter I

Introduction

Approximately 1,000 prenylated polyphenols have been isolated and identified [1-3], among which glyceollins (prenylated 6a-hydroxy-pterocarpans) are a typical metabolite of soybean isoflavones [4]. Glyceollins are also called as phytoalexin, since they are produced in soybeans from isoflavones by environmental (*e.g.,* infection, wounding, freezing, ultraviolet light) or microbial stresses [5-7]. The metabolism of isoflavones in soybean seed has been extensively investigated; more than 7 species of glyceollins (I, II, III, IV, V, VI, and glyceofuran) have been identified in stressed soybean so far [8, 9], and upregulation of regiospecific prenyltransferases (PTs), *e.g.,* (-)-glycinol 2-dimethylallyltransferase (G2DT), and (-)-glycinol 4-dimethylallyltransferase (G4DT) are involved in the prenylation reaction in soybean seed [5]. The biosynthetic pathways of glyceollins (I, II, III) from isoflavones [8] are represented in **Fig. 1-1**.

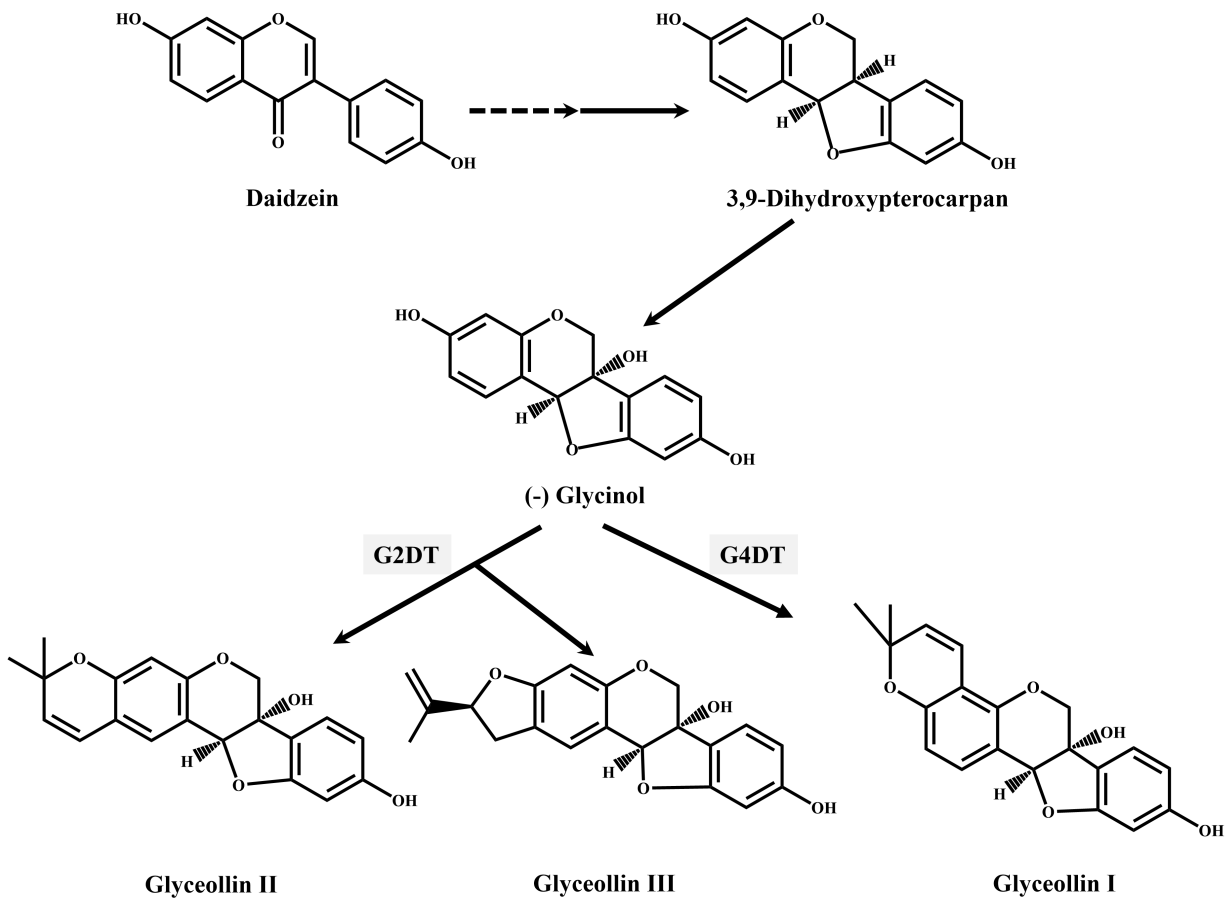


Fig.1-1 A biosynthesis of prenylated isoflavones, glyceollins (I, II, III), from daidzein in soybean under stressed conditions.

Abbreviations: G2DT, (-)-glycinol 2-dimethylallyltransferase; G4DT, (-)-glycinol 4-dimethylallyltransferase.

Clinical evidence reveals that glyceollins have diverse *in vivo* physiological functions, such as antioxidation [10], anti-diabetes [11], anti-inflammation [12], and antagonistic effect against estrogen receptor (ER) [13]. Since glyceollins (I, II, and III), as well as more stable phytochemical, 6a,11a-pterocarpenes, possess structural similarity to the female sexhormone 17β-estradiol (E2) [14], current research lies on their ER-mediated anti-tumour effect. Burow *et al.* have reported that a mixture of glyceollins suppressed the E2-induced proliferation of ER-positive human breast cancer MCF-7 cells [15]. Glyceollins also showed a 3-fold higher affinity for ERα than for ERβ in a competition binding assay [16]. On the other hand, it has been reported that glyceollin I exerted potent anti-proliferative effect by inhibiting the Eleanor RNA cloud and ERα mRNA transcription in long-term estrogen-deprived cells [17]. A subsequent study revealed that glyceollin I exhibited the strongest anti-osteogenic activity compared to glyceollins II and III [18], and downregulated E2-responsive stromal cell-derived factor-1 and progesterone receptor expression in breast cancer MCF-7 and BG-1 cells [13]. In addition to the anti-tumour effects, some health benefits of glyceollins are reported in literature (**Table 1-1)**.

Table 1-1 Health benefits of glyceollins *in vivo* and *in vitro*

health claim	compound	cell /animal	mechanism	reference
neuromodulation	glyceollins	breast cancer MCF-7 cell	increased nerve growth factor receptor gene expression	[13]
	glyceollins	glutamate-sensitive murine hippocampal HT22 cell	attenuated neurotoxicity; suppressed intracellular ROS; actived heme oxygenase-1 enzyme	[19]
	glyceollins	wide-type and Nrf2 knockout C57BL/6J mouse	suppressed cytotoxicity and improve cognitive in wide-type mouse	[19]
	glyceollins	ovariectomized adult CFW mouse	upregulated/downregulated genes through neurodegeneration apoptosis and transcripts	[14, 20]
anti-inflammatory	glyceollins	murine macrophage RAW264.7 cell	inhibited nitric oxide production and inflammatory mediator release	[12, 21]
	glyceollins	human umbilical vein endothelial cell	reduced HMGB1 protein level induced by LPS	[22]
	glyceollins	ICR mouse	reduced mouse ear swelling	[12]
	glyceollins	C57BL/6 mouse	reduced serum HMGB1 level	[20]
anti-cardiovascular	glyceollins	human aortic smooth muscle cell	inhibited cell proliferation and migration	[23]
	glyceollin I	aortic rings from SD rat	reduced vascular contraction	[24]
anti-bacterial	glyceollins	B16/B16F10 cell	inhibited melanin synthesis	[25, 26]
	glyceollins	standard AB strain zebrafish embryos	decreased the pigmentation of the embryos, melanin synthesis and tyrosinase activity	[26]

health claim	compound	cell /animal	mechanism	reference
anti-osteoporosis	glyceollins I and II	adipose-derived stromal cell bone marrow stromal cell	increased calcium deposition	[27]
anti-diabetic	glyceollins	enteroendocrine NCI-H716 cell	enhanced GLP-1 secretion	[28]
	glyceollins	3T3-L1 adipocyte cell	increased glucose transporter GLUT4 level	[11, 28]
	glyceollins	insulima Min6 cell	decreased apoptosis and enhanced insulinotropic actions	[28]
	fermented soybeans containing glyceollins	diabetic C57BL6J mouse	decreased blood glucose level and increased hepatic glycogen accumulation	[29]
	glyceollins	prediabetic ZDSD/Pco rat	decreased blood glucose level	[11]
	diet containing glyceollins	golden Syrian hamster	altered expression of cholesterol genes in liver	[30]
	diet containing glyceollins	monkey	decreased serm total cholesterol and upregulated gene expression	[31]
anti-oxidative	glyceollins	*in vitro*	ferric-reducing antioxidant power, radical scavenging activities	[10]
	glyceollins	hepa1clc7/BRPc1 cell	inhibited H_2O_2-induced ROS production and activated Nrf2-signaling pathway	[10 19, 32]
	glyceollins	C57BL6J mouse	decreased NAD(P)H oxidase	[33]
	glyceollins	SD rat	inhibited lipid peroxidation	[10]

Irrespective to physiological potentials of glyceollins, it is crucial to understand their absorption, distribution, metabolism, and excretion (ADME) in the body system. Therefore, not only the analysis of intestinal absorption and metabolism, but also the analysis of accumulation in the organs, should be clarified for deeper understanding of bioactive glyceollins [2, 34-36].

For the bioavailability of isoflavones, as a precursor of glyceollins, genistein and daidzein, as well as their conjugates, has already been reported in literature [37-39]. Target distribution organs of ferulic acid (4-hydroxy-3-methoxy cinnamic acid) [40, 41], caffeic acid (3, 4-dihydroxy-cinnamic acid) [42, 43], naringin [44], and daidzein [45, 46] in Sprague-Dawley (SD) rats are shown in **Table 1-2**.

Table 1-2. Distribution of phenolic acids and polyphenols after administration in Sprague-Dawley rats in literature

compounds	metabolite form	blood	organ					reference
			liver	kidney	heart	lung	soleus muscle	
ferulic acid	Sulfated Glucuronized	○	○	○	○	○	not available *(n.a.)*	[40, 41]
caffeic acid	Sulfated Glucuronized Methylated Sulfated/glucuronized	*n.a.*	○	○	○	○	*n.a.*	[42, 43]
naringin	Glucuronized Diglucuronized Sulfated Disulfated Glucuronized/sulfated	○	○	○	○	○	○	[44]
daidzein	Equol *cis*-4-Hydroxylequol Dihydroxylated Tetrahydroxylated	○	○	○	○	○	*n.a.*	[45, 46]

For the reports about the influence of prenylation on the bioavailability of polyphenols, it has been revealed that prenylation improved cellular uptake of quercetion [35] and naringenin [47], as well as the accumulation of 8-prenyl quercetion and its metabolites in the muscle and the kidneys [35] (**Fig. 1-2**). Prenylation also enhanced the estrogenicity of naringenin and genistein [34], as well as elevated inhibitory effect of prenylated luteolin on melanin biosynthesis in cell cultures [48]. For prenylated isoflavones, glyceollins, current study mainly reported the metabolism and pharmacokinetics in blood; a glyceollin-enriched diet [glyceollin I (76.8%), II (9.9%), and III (13.6%)], with a daily intake in 12.57 mg/kg/day for 14 days to monkeys resulted in a detection in plasma at 16.7–324.3 nmol/L [49]. An oral administration of a mixture of glyceollin I (68%), II (21%), and III (11%) to ZDSD rats confirmed the absorption in blood at 0.24–0.35 μmol/L and 0.35–0.47 μmol/L at a dose of 30 mg/kg and 90 mg/kg, respectively [11]. Since the impact of prenylation of isoflavones on bioavailability is not clear, therefore, we aim to clarify bioavailability, intestinal absorption and metabolism, and tissue accumulation, in the current study.

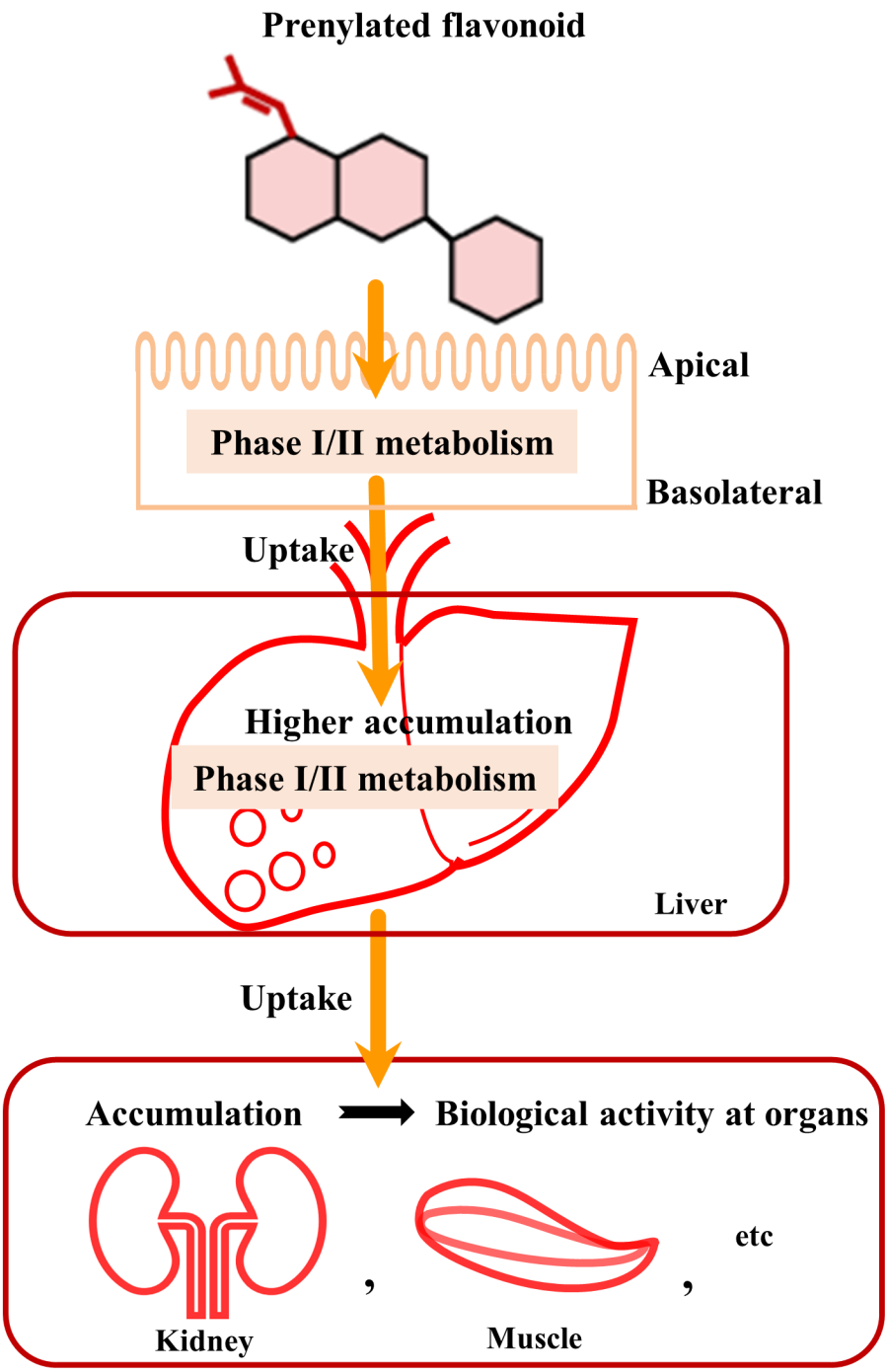


Fig.1-2 Intestinal absorption and tissue accumulation of prenylated flavonoids.

The first step of intestinal absorption determines the magnitude of bioavailability, and the absorbability is dependent on the balance between influx and efflux events in intestinal epithelial cells [50]. Caco-2, a human colorectal adenocarcinoma cell line, is extensively used as a model of small intestinal epithelium because of the advantage of having influx/efflux routes [50]. For influx route, the isoflavones, as a precursor of glyceollins, genistein and daidzein, transported across Caco-2 cells mainly dominated by transcellular and paracellular routes [51], while the influx route of glyceollins in the intestinal membrane remain unclear. For efflux route, ATP-binding cassette (ABC) transporters, such as multidrug-resistant protein 2 (MRP2) and breast cancer-resistant protein (BCRP), were implicated in the efflux of glyceollins in Caco-2 cells [52-54]. In addition, glyceollins appear to inhibit the metabolism of the parent isoflavone of genistein during the intestinal transport process [55]. These findings allow us to further investigate the intestinal absorption and metabolism of glyceollins.

Once being absorbed into the circulation system or the organs, flavones may be hydrolysed to aglycons by lactase-phlorizin hydrolase and microflora in the gut, subsequently encountering phase I/II metabolism [56]. Namely, methylation, sulfation, and glucuronidation, catalyzed by phase II enzymes named catechol-*O*-methyltransferase (CMT), sulfotransferase (SUT), and uridine diphosphate-glucuronosyltransferase (UDGT), respectively, are involved in phase II metabolism [57]. It appears that equol (7-hydroxy-3-(4′-hydroxyphenyl)-

chroman), an isoflavone (daidzein) metabolite generated in the gut by intestinal bacteria, may be a candidate responsible for the improvement bone health in ovariectomized rats by trabecular microarchitecture increase in the proximal femur and lumbar spine [58]. The physiological potentials of sulfated metabolites, such as isovanillic acid 3-*O*-sulfate (IVAS) exhibited an ability to stimulate a glucose transport via the GLUT4 [59] and 4-*O*-sulfated ferulic acid showed a potent anti-hypertensive action in mice [60]. These findings allowed us to investigate the physiological potential of glyceollin conjugates in the accumulation organs.

Following absorption and metabolism, pharmacological activity in target organs is dependent upon tissue distribution. It has been reported that isoflavones (genistein and daidzein), with a daily intake in 13.6–17.3 mg/kg/day for 3 weeks, were detected in intact form in the liver at 0.26–0.65 nmol/g-wet tissue for male SD rats [61]. Conjugated forms of glucuronized and sulfated genisteins were also detected in the liver [62] and the placenta of SD rats [63]. Urpi-Sarda *et al*. reported that a daily intake of genistein (7.6 mg/kg) or daidzein (2.9 mg/kg) in ewes for 1 month caused an apparent tissue accumulation of intact and conjugated isoflavones in the blood, the liver, the kidneys, the aorta, the lungs, the heart, and the muscle; particularly, daidzein accumulation in the kidney reached 71.23 nmol/g-tissue [64]. Moreover, the elevated hepatic insulin sensitivity of glyceollins in the liver of diabetic mice [29] strongly suggested that they may play a physiological role in organs, though no accumulation reports are available.

Possible strategies for evaluating the bioavailability of prenylated isoflavones, glyceollins in this study, are described below.

Strategy for the analysis of intestinal absorption behavior of prenylated isoflavones, glyceollins

Intestinal absorption of phytochemicals may be involved in passive transport (transcellular/paracellular diffusion) and active transport (influx/efflux transport), presumably depending on their structural and/or chemical characteristics [65]. The intestinal absorption of glyceollins [52-54], as well as their parent isoflavones (daidzein [66] and genistein [67]), have been widely investigated by cell-based *in vitro* models. Irrespective to easy set of cell-line experiments, Caco-2 cell model remains disadvantages, such as different protease expression from animal intestinal membrane. An alternative strategy for absorption study has been proposed by *ex vivo* Ussing Chamber system, which is mounted with animal intestinal membrane [68]. The *ex vivo* system, which is a good tool for investigating transport mechanism as similar to *in vivo* intestinal absorption events, is successfully used for transport of polyphenols [69] and peptides [70].

Strategy for the analysis of tissue accumulation of prenylated isoflavones, glyceollins

Pharmacokinetics of glyceollins have been studied in rats [11] and monkeys [49]. However, accumulation study of orally administered glyceollins in circulatory organs is limited. A crucial question is whether specific organs are

involved in the accumulation of glyceollins. Thus, quantitative evaluation of glyceollins in target organs would provide useful information for assessing their mechanisms of action. Thus far, a prenyl flavonoid, 8-prenyl kaempferide, was detected at the liver, the spleen, the muscle, the lung, the kidneys, and the heart as intact and conjugated forms [71]. 8-Prenyl naringenin was also reported to be accumulated in the muscle at 10 times higher concentration than that of the parent naringenin [47]. Similarly, 8-prenyl querctin and *O*-methylated form were accumulated in the liver and the kidneys at higher levels than that of querctin in male C57/BL6 mouse [35]. These accumulation studies suggest that prenylation could promote tissue accumulation. However, there is no study about the accumulation of glyceollins of prenylated isoflavones, together with their metabolic behavior.

Thus, the aim of this study is to obtain insights of bioavailability of prenylated isoflavones, glyceollins, in SD rats. The objectives for each Chapter in this study are described as follows:

1) In **Chapter II**, in order to clarify the characteristics of intestinal absorption of prenylated isoflavones, glyceollin I and glyceollin III were used for oral administration to SD rats. Glyceollin metabolism during the absorption in rat intestinal membrane was also evaluated by combinatorial mass spectrometry (MS) techniques using matrix-assisted laser desorption/ionization (MALDI)-MS and liquid chromatography time-of-flight (LC-TOF)/MS.

2) In **Chapter III**, accumulation behavior of glyceollin I and glyceollin III in the organs of SD rats was evaluated by LC-TOF/MS. In this study, the liver, the kidneys, the heart, the lungs, the soleus muscle, and the abdominal aorta of SD rat were targeted.

Taken together all, the present study will provide new insights of pharmacokinetic advantages of prenylated polyphenols in terms of absorption efficacy and distribution preference into organs.

Chapter II

Intestinal absorption of prenylated isoflavones, glyceollins, in Sprague-Dawley rats

1. Introduction

It has been reported that glyceollins (prenylated 6a-hydroxy-pterocarpans), a variety of isoflavone metabolites (called as phytoalexins), are produced in soybean in response to environmental (*e.g.,* infection, wounding, freezing, ultraviolet light) or microbial stress [1, 4]. The characterized prenylated moieties occur in the structure of isoflavones, daidzein and genistein [5]. Apart from the metabolic conversion mechanism of isoflavones to glyceollins in soybean, current research concerns their physiological functions [7]. For example, glyceollin mixture inhibited the 17β-estradiol (E2)-induced proliferation of ER-

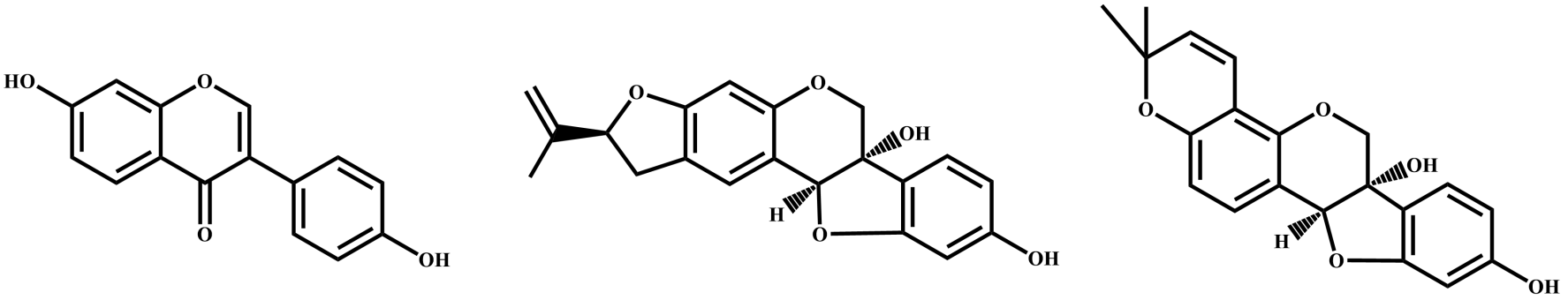
positive human breast cancer MCF-7 cells [15]. Besides, glyceollin I exerted a potent anti-proliferative effect in recurrent breast cancer cells by inhibiting the Eleanor RNA cloud and ERα mRNA transcription [17]. These benefits for glyceollins, but not for their parent isoflavones, lead us to investigate their bioavailability or the magnitude of absorption, since glyceollin absorption remains unresolved.

It is known that isoflavones (genistein and daidzein) undergo phase I degradation and/or phase II metabolism (sulfated, glucuronided, and methylated conjugates) during intestinal absorption process [72]. The daidzein metabolite in the gut by intestinal bacteria, equol (7-hydroxy-3-(4′-hydroxyphenyl)-chroman), may be a candidate responsible for the effect [73]. A good example is that the equol supplementation was associated with bone health in ovariectomized rats by altered trabecular microarchitecture at the proximal femur and lumbar spine as well as osteoporotic fracture healing [58]. Taken together, in **Chapter II**, daidzein (as comparable isoflavone), glyceollin III, and glyceollin I administered to rats by oral to make clear the absorption characteristics of glyceollins. A combinatorial mass spectrometry (MS) techniques using MALDI-MS and LC-TOF/MS were also conducted to identify the glyceollin metabolites during the intestinal absorption process.

2. Materials and methods

2.1. Materials

Glyceollin III and glyceollin I were prepared according to the reported procedures [17, 74]. Briefly, *Aspergillus orzae*-stimulated soybeans (20 kg) were extracted with 80 L of *n*-hexane. After vacuum filtration, the residue was extracted with 80 L of 70% ethanol (EtOH). The filtered concentrate was then injected to an HP-20 column (35 cm i.d. × 50 cm; Mitsubishi Chemical Co.; Tokyo, Japan) to obtain glyceollin III (yield: 1 mg/kg) and glyceollin I (yield: 9 mg/kg). The structures were determined by nuclear magnetic resonance (NMR) and MS spectrometry, as depicted in **Fig. 2-1.** Daidzein and 1,5-diaminonaphtalene (1,5-DAN) were purchased from Tokyo Chemical Ind. Co. (Tokyo, Japan). Taxifolin was purchased from TCI Fine Chemicals (Tokyo, Japan). Sulfatase type H-1 (EC 3.1.6.1, 25 units, from *Helix pomatia*) and β-glucuronidase type B-1 (EC 3.2.1.31, 50 units, from bovine liver) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Milli-Q water was prepared using a Milli-Q system (Millipore, Tokyo, Japan). Commercial distilled water, methanol (MeOH), EtOH, acetonitrile (ACN), dimethyl sulfoxide (DMSO) and formic acid (FA) were of MS grade (Merck, Darmstadt, Germany). All other chemicals were of analytical reagent grade and used without further purification.



	Daidzein (exact mass, 254.0506)	Glyceollin III (exact mass, 338.1081)	Glyceollin I (exact mass, 338.1081)
Log *P* :	2.632 ± 1.134	3.60 ± 0.515	3.907 ± 0.493

Fig. 2-1 Chemical structures of daidzein, glyceollin III, and glyceollin I used in this study.

Molecular weight and hydrophobicity (log *P* value) of each molecule were available from a SciFinder Substance Identifer software (https://scifinder.cas.org/scifinder/view/scifinder/ scifinderExplore.jsf).

2.2. A single oral administration experiment of glyceollins in Sprague-Dawley rats

Seven-week-old male SD rats (SPF/VAF Crj; Charles River Japan, Kanagawa, Japan) were fed on a laboratory diet (CE-2, Clea Japan, Tokyo, Japan) and given water *ad libitum*. All rats were housed for 1 week under the temperature of 21 ± 1℃ and the humidity of 55 ± 5%. Each rat was starved for 16 h before a single oral administration of daidzein, glyceollin III, or glyceollin I dissolved in 0.5% carboxymethyl cellulose (CMC) by gavage (1.0 mL of each sample at a dose of 1.0 mg/kg body weight). At a time-point up to 8 h (0, 0.5, 1, 4, and 8 h), an aliquot of blood (200 μL) was collected from the tail vein and centrifuged at 3,500 × *g* for 15 min at 4°C to obtain plasma. Plasma samples were stored at –40°C until analysis. All animal experiments were carried out in accordance with the guidelines set by the Guidance for Animal Experiments in the Faculty of Agriculture and in the Graduate Course of Kyushu University in accordance with the Law (No. 105, 1973) and Notification (No. 6, 1980, of the Prime Minister's Office and No. 71, 2006, of the Ministry of Health, Labor and Welfare) of the Japanese Government. All experimental protocols were reviewed and approved by the Animal Care and Use Committee of Kyushu University (permit number: A20-095).

2.3. Preparation of rat plasma for LC-TOF/MS analysis

For the determination of daidzein, glyceollin III, and glyceollin I in rat plasma, 50 μL of plasma samples were mixed with an equivalent volume of 100 mM sodium acetate

buffer (pH 4.0), following the addition of 10 μL of 5.0 μM taxifolin as internal standard (IS, final concentration: 1.0 nmol/mL). Alternatively, for the determination of conjugates, the prepared solution (110 μL) was subjected to an enzymatic treatment (12 h, 37°C) by adding 20 μL sulfatase type H-1 (25 units) and 10 μL β-glucuronidase type B-1 (50 units [75]). Solutions with or without enzymatic treatment were then mixed with 100 μL of EtOH, centrifuging at 14,000 × *g* for 10 min at 25°C. The supernatant was applied on an Amicon Ultra 0.5-mL 3K centrifugal filter (Millipore, Carrigtwohill, Ireland, 14,000 × *g*, 25°C, 30 min). The obtained filtrate was evaporated to dryness, and then dissolved in 50 μL of 50% EtOH prior to LC-TOF/MS analysis.

2.4. LC-TOF/MS analysis

LC separation was performed using an Agilent 1200 series (Agilent; Waldbronn, Germany) on a Cosmosil 5C_{18}-MS-II column (2.0 × 150 mm, Nacalai Tesque; Kyoto, Japan) at 40°C with a linear gradient elution of 0.1% FA (solvent A) to MeOH containing 0.1% FA (solvent B) over 80 min at a flow rate of 0.25 mL/min. Daidzein ($[M - H]^-$: 253.0506 *m/z*), glyceollin III ($[M - H]^-$: 337.1081 *m/z*), and glyceollin I ($[M - H]^-$: 337.1081 *m/z*) were analyzed with a micrOTOF II mass spectrometer (Bruker Daltonics; Bremen, Germany) in negative electrospray-ionization (ESI) mode. Mass spectral data were collected within the range of 100–1000 *m/z*. MS conditions were as follows: nebulizer pressure, 1.6 bar; dry gas, nitrogen; dry gas flow rate, 8.0 L/min; drying temperature, 200°C; HV capillary voltage, –4,500 V; capillary exit, –130.0 V. The calibration solution

of 10.0 mM sodium formate in 50% ACN was injected at the each beginning of the run. Data were analyzed using a Bruker Data Analysis version 4.0 software. Quantification of targets in rat plasma by LC-TOF/MS were quantified using calibration curves of y = 8.7136x + 0.0641 [r^2 = 0.9949; limit of detection (LOD), 1.0 pmol/mL] for daidzein, y = 2.0951x – 0.1307 [r^2 = 0.9891; limit of detection (LOD), 1.1 pmol/mL] for glyceollin III, and y = 4.8161x – 0.714 [r^2 = 0.992; limit of detection (LOD), 0.8 pmol/mL] for glyceollin I, where y is the ratio of observed peak area against that of IS and x is the concentration (nmol/mL).

2.5. Transport experiments using rat jejunum membrane

Transport experiments of glyceollins using SD rat jejunum membrane were performed in a Ussing chamber system (Dual Channel Ussing Chamber; model U-2500; Warner Instrument; Hamden, CT, USA), as described in previous report [69]. Shortly, a jejunum segment (15–20 cm below the stomach) was washed with Krebs-Bicarbonate Ringer's solution (KBR, pH 7.4, 2.5 mM $CaCl_2 \cdot 2H_2O$, 4.8 mM KCl, 1.3 mM KH_2PO_4, 1.2 mM $MgSO_4 \cdot 7H_2O$, 118.1 mM NaCl, 10 mM d-glucose, and 25 mM $NaHCO_3$) and then cut along the mesenteric border to expose the mucosal side. The prepared segment was mounted in the Ussing chamber, followed by a 60-min transport experiment of either 100 μM daidzein, glyceollin III, or glyceollin I. After the 60-min transport, 4 mL of basolateral solution was collected, evaporated and dissolved in 200 μL of DMSO containing 1.0 nmol/mL taxifolin. The uptake (pmol/mL) of each target in the basolateral solution was

determined by the above-mentioned LC-TOF/MS using the abovementioned calibration curves. Alternatively, the 60-min transported intestinal membranes were lyophilized after washing thrice with KBR solution for further metabolite analyses by LC-TOF/MS and MALDI-MS.

2.6. Analyses of glyceollin metabolites in rat jejunum membrane by MALDI-MS and LC-TOF/MS

The 60-min transported intestinal membranes were mashed with a BioMasher II (Nippi. Inc.; Tokyo, Japan) and 1.0 mg of powdered homogenate was dissolved in 1.0 mL of 70% EtOH. The sample solution was then sonicated using a SONIFIRE 250 (Branson Ultrasonics; Emerson Japan Co.; Kanagawa, Japan) with an output control of 3 for 10 s thrice at 4°C, and then homogenized with a Polytron homogenizer (Kinmatica AG; Luzern, Switzerland) at 20,000 rpm for 30 s twice. After the centrifugation of the homogenate at 14,000 × *g* for 15 min at 4°C, the supernatant was ultra-filtered using an Amicon Ultra 0.5-mL 3K centrifugal filter at 14,000 × *g* for 30 min at 4°C. The filtrate was evaporated to dryness and then dissolved in 30 μL of 50% EtOH for both the aforementioned LC-TOF/MS and MALDI-MS analyses.

MALDI-MS analysis of the filtrate was conducted with an Autoflex III MS equipped with SmartBeam III (Bruker Daltonics) in negative ion-linear mode. An aliquot (0.2 μL) of the filtrate sample was spiked onto an indium-tin oxide (ITO)-coated glass slide and

then sprayed with 1,5-DAN (10 mg/kg in 70% ACN) using an ImagePrep automatic matrix sprayer (Bruker Daltonics). MS data was acquired in the range of 100–1000 *m/z* by averaging signals from 2,500 laser pulses. MALDI-MS parameters were as follows: ion source 1, 20.00 kV; ion source 2, 18.80 kV; lens voltage, 7.50 kV; gain factor, 10.50; laser frequency, 200 Hz; laser power 40%; of set, 59%; range, 20%; laser focus range, 100%; and value, 6%.

2.7. Statistical analysis

Pharmacokinetic analysis of concentration-time data was performed using a GraphPad Prism software (GraphPad; La Jolla, CA, USA). The maximum plasma concentration (C_{max}), time of maximum concentration (T_{max}), elimination half-life ($T_{1/2}$), and the area under the curve up to 8 h ($AUC_{0\text{-}8\ h}$) were obtained directly from the plasma concentration-time plots. Results are expressed as the mean ± standard deviation (S.D.).

3. Results and discussion

3.1. Absorption of glyceollins in Sprauge-Dawley rats

The effect of isoflavone prenylation on intestinal absorption compared to that of daidzein (a parent isoflavone) was investigated by single oral administration experiments

of glyceollins (glyceollin III and glyceollin I), in SD rats at each dose of 1.0 mg/kg. Daidzein, which is a precursor of glyceollins [5], was also orally administered to SD rats (1.0 mg/kg) as comparative control. Considering the average amount of soybean intake (76 –165 mg/day in Japan [76]) and the content of isoflavones in soybean (1176–1749 μg/g of soybean [77]), the dose (1.0 mg/kg) of glyceollins and daidzein in this study would be acceptable. As shown in **Fig. 2-2A**, the limits of detection (LOD) for daidzein, glyceollin III, and glyceollin I in the present LC-TOF/MS were: 1.0 pmol/mL, 1.1 pmol/mL, and 0.8 pmol/mL, respectively. No MS peak at 337.1081 *m/z* corresponding to each glyceollin at corresponding LC retention time was observed, as well as 253.0506 *m/z* for daidzein. This implied that glyceollin III and glyceollin I, as well as daidzein, are not absorbed into rat circulating bloodstream in their intact forms in the present animal experiments. As isoflavones, such as daidzein and genistein, were reported to convert in the gut and in the intestine to form *e.g.*, equol in the gut and sulfated and/or glucuronized [78], experiments were performed for conjugated forms of glyceollins with the aid of β-glucuronidase Type B-1 and sulfatase Type H-1 deconjugation treatments [75]. As shown in **Fig. 2-2B**, it appeared that the deconjugation treatment of plasma by both the enzymes resulted in an apparent appearance of MS peaks corresponding to daidzein ($[M - H]^-$: 253.0506 *m/z*), glyceollin III ($[M - H]^-$: 337.1081 *m/z*), and glyceollin I ($[M - H]^-$: 337.1081 *m/z*); some glyceollins may be metabolized to sulfated and/or glucuronized conjugates, as similar to isoflavones metabolism before entering the circulating bloodstream [79, 80].

Equol, which is a reported isoflavone metabolite in the gut by intestinal bacteria [73], as well as its possible metabolite, hydroxyl equol (equol-OH) [58], was not detected under the present experimental conditions (1.0 mg/kg dose). In contrast, equol-OH ($[M - H]^-$: 257.0819 *m/z*) was detected after enzymatic deconjugation treatments (**Fig. 2-3**). A successful MS detection of equol-OH after enzymatic deconjugation treatments indicates that the ingested daidzein was in part converted to equol, followed by hydroxylation in phase I metabolism [73] and subsequent conjugation by phase II metabolism [81] during the absorption process. No MS detection of equol ($[M - H]^-$: 241.0870 *m/z*) might be due to low ESI-ionization efficiency of equol [limit of detection (LOD), 27.8 pmol/mL] or rapid conversion of equol to equol-OH. In addition, the MS peaks corresponding to *m/z* values of conjugates (sulfated and/or glucuronized forms) for glyceollin III and glyceollin I were also observed in plasma at a low dose of 1.0 mg/kg (**Fig. 2-4**). This suggests that the reported physiological actions of other isoflavones, such as anti-tumor effect of 8-prenylgenistein [34], 8-hydroxy glycitein [82], and glyceofuran [83] might be caused by their metabolic forms as glyceollins.

As shown in **Fig. 2-5**, a single oral administration of glyceollins (1.0 mg/kg) to SD rats led to a rapid absorption in conjugated forms in blood with a T_{max} of 0.5 h. However, a 24 h-assay of glyceollins in blood may be needed to clarify their detailed pharmacokinetics, since glyceollins were detected in blood in conjugated forms at 8 h after single oral administration ($T_{1/2}$: glyceollin III, 4.7 h; glyceollin I, 3.1 h) (**Fig. 2-4**). The absorption amount of glyceollins was greatly different from the structure. As summarized

in **Table 2-1**, glyceollin I as conjugated form ($AUC_{0–8\,h}$: 8.5 ± 0.7 nmol·h/mL-plasma) was much higher than that of glyceollin III ($AUC_{0–8\,h}$: 1.0 ± 0.2 nmol·h/mL-plasma), and > 14-times higher than that of its parent isoflavone, daidzein ($AUC_{0–8\,h}$: 0.6 ± 0.1 nmol·h/mL-plasma). Compared to the reported absorption of hesperidin (hesperetin-7-*O*-rutinoside), in which the absorption as conjugated form was $AUC_{0\text{-}24\,h}$ of 6.4 ± 0.9 nmol·h/mL-plasma (10 mg/kg dose) [75], the higher absorption of glyceollin I was obtained at lower dosage (1.0 mg/kg). Although Boué *et al*. reported that when a mixture of glyceollins I (68%), II (21%), and III (11%) was administered to male ZDSD/Pco rats at a dose of 30 mg/kg, the overall absorption of glyceollins had a C_{max} of 0.35 ± 0.05 nmol/mL [11]. In this study, the absorbability of glyceollins was clarified in the descending order of glyceollin I (C_{max}: 1.9 ± 0.6 nmol/mL) > glyceollin III (C_{max}: 0.25 ± 0.05 nmol/mL) > daidzein (C_{max}: 0.11 ± 0.03 nmol/mL) (**Table 2-1**). Epigallocatechin-3-*O*-gallate (EGCG), 100 mg/kg [84] and cyanidin-3-*O*-glycoside (C3G), 45 mg/kg [85] were reported to be an absorbable phytochemical, showing a C_{max} of 1.23 ± 0.16 nmol/mL and 2.4 ± 0.5 nmol/mL in plama for EGCG and C3G, respectively. While, the C_{max} of both compounds were much higher than those of glyceollins, the absorption efficiency of glyceollins per dose (C_{max}/dose of glyceollin I, 1.9 nmol/mL/mg-dose; glyceollin III, 0.25 nmol/mL/mg-dose) was predominant rather than those of EGCG (C_{max}/dose: 0.0123 nmol/mL/mg-dose), C3G (0.053 nmol/mL/mg-dose), daidzein (0.11 nmol/mL/mg-dose, **Table 2-1**) and genistein (0.070 nmol/mL/mg-dose [86]).

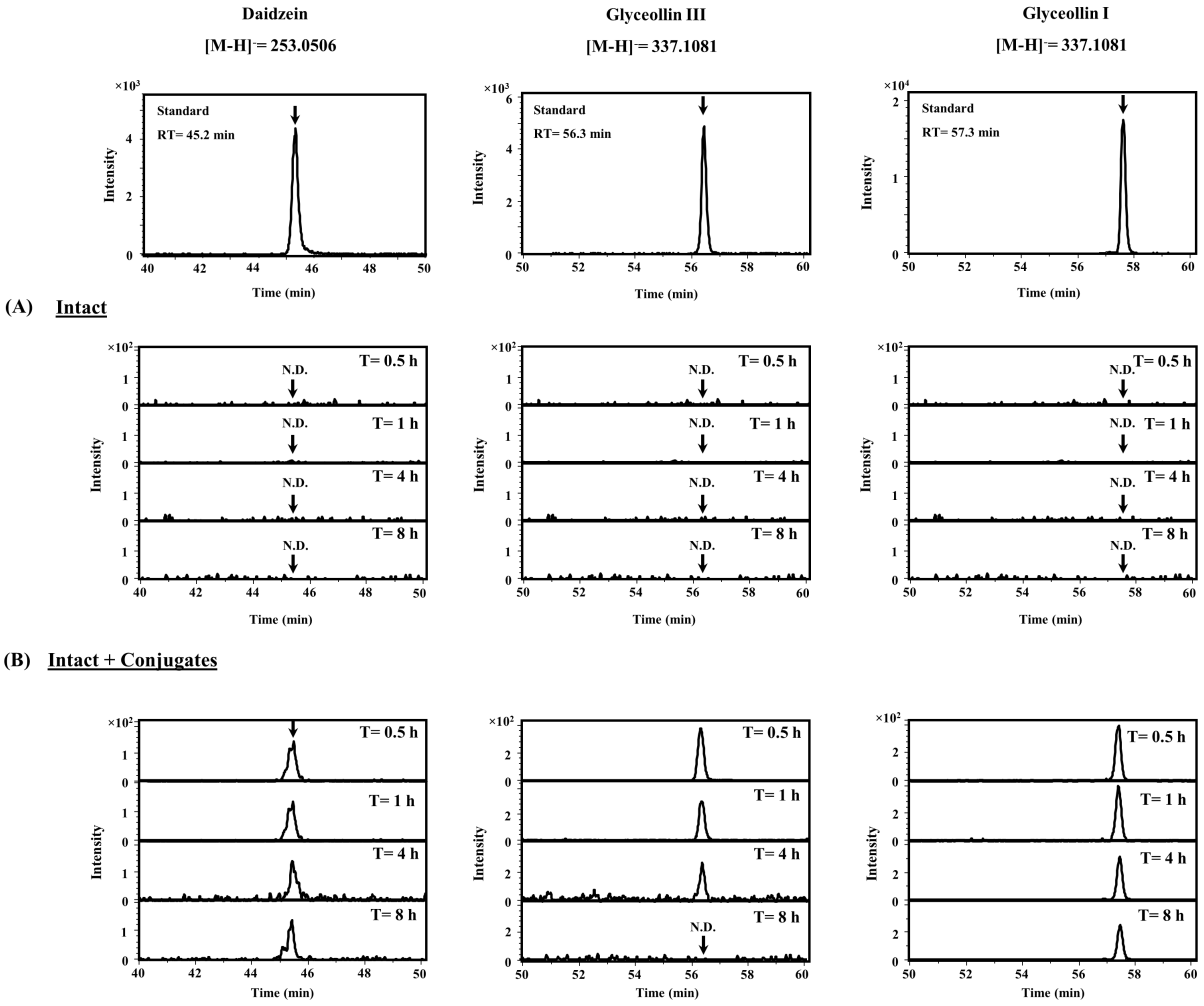


Fig. 2-2 LC-TOF/MS chromatograms of daidzein, glyceollin III, and glyceollin I in circulating plasma of SD rats collected at 0.5, 1, 4, and 8 h after oral administration (1.0 mg/kg).

Concentration of each standard was 1 μM. MS chromatograms show the elution of each target in plasma: (A) intact, (B) intact + conjugates. N.D. indicates no detection.

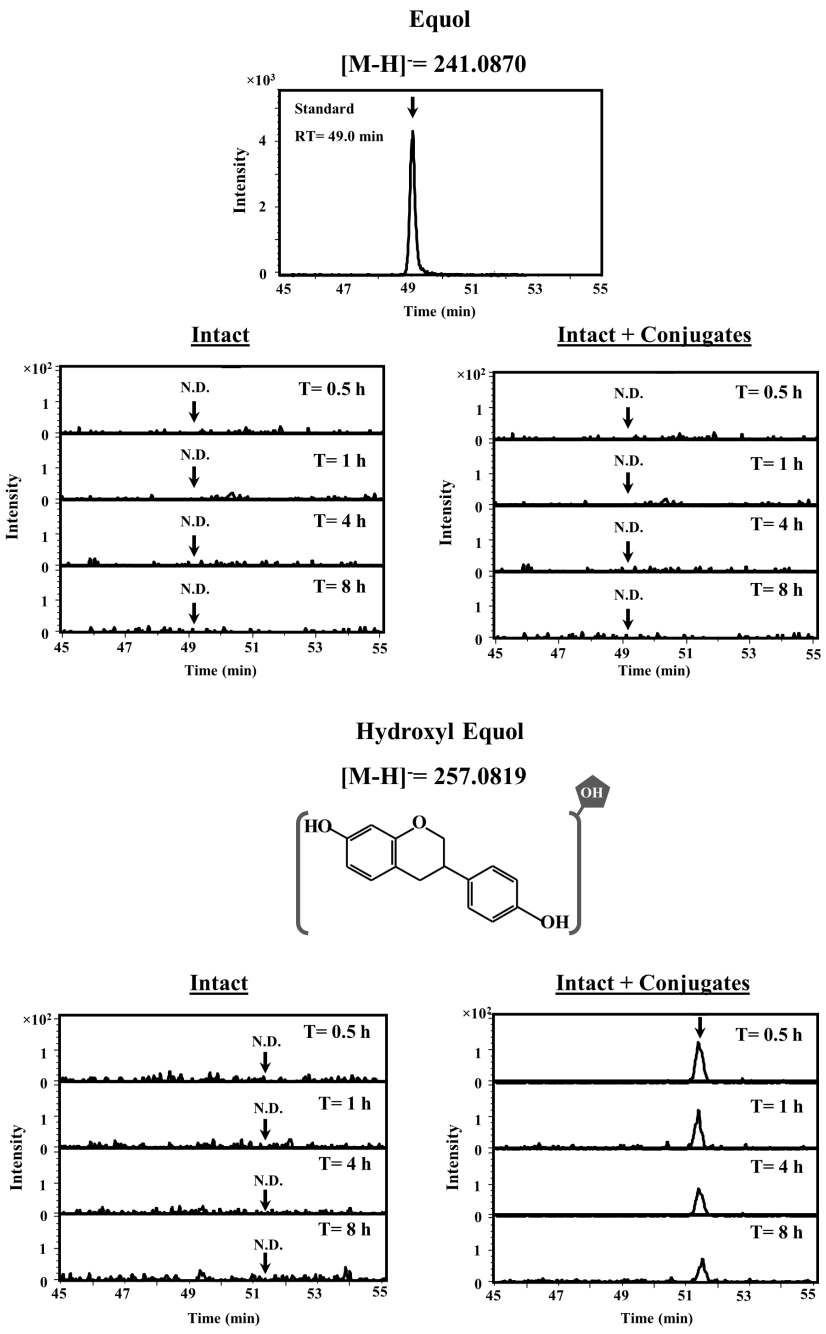


Fig. 2-3 LC-TOF/MS chromatograms of equol and hydroxyl equol in circulating plasma of SD rats after daidzein administration to SD rats (1.0 mg/kg).

Concentration of equol standard was 5 μM. MS targeted equol and hydroxyl equol were detected by EIC-LC-TOF/MS with their *m/z* values (equol: $[M - H]^-$=241.0870 *m/z*; hydroxyl equol: $[M - H]^-$=257.0819 *m/z*). N.D. indicates no detection.

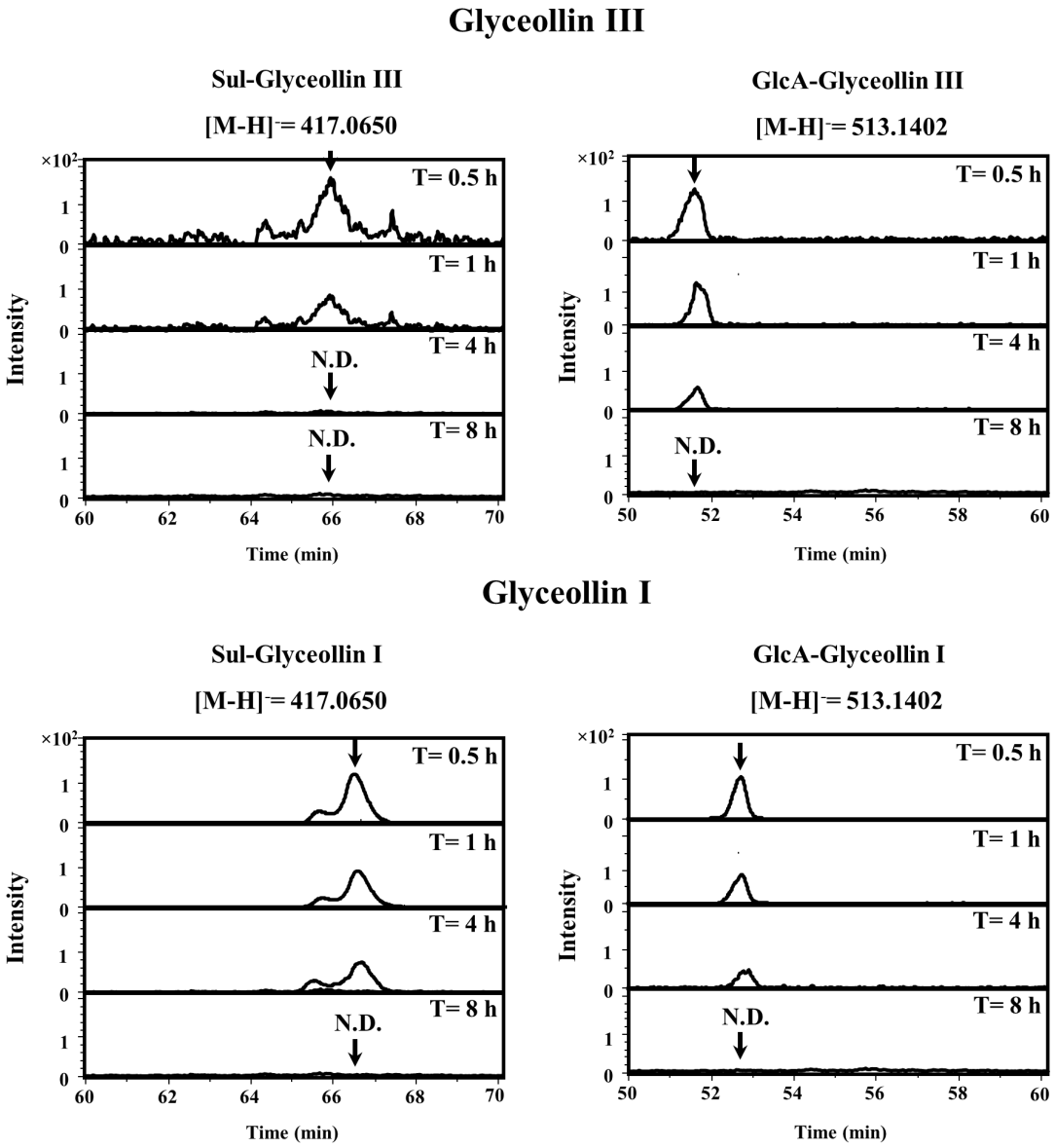


Fig. 2-4 LC-TOF/MS chromatograms of glyceollin III and I conjugates in circulating plasma of SD rats after glyceollin III/I administration to SD rats (1.0 mg/kg).

MS targeted Sul-glyceollin III/I and GlcA-glyceollin III/I were detected by EIC-LC-TOF/MS with their *m/z* values (Sul-glyceollin III/I: $[M - H]^-$=417.0650 *m/z*; GlcA-glyceollin III/I: $[M - H]^-$ =513.1402 *m/z*). N.D. indicates no detection. Abbreviations: GlcA (glucuronided), Sul (sulfated).

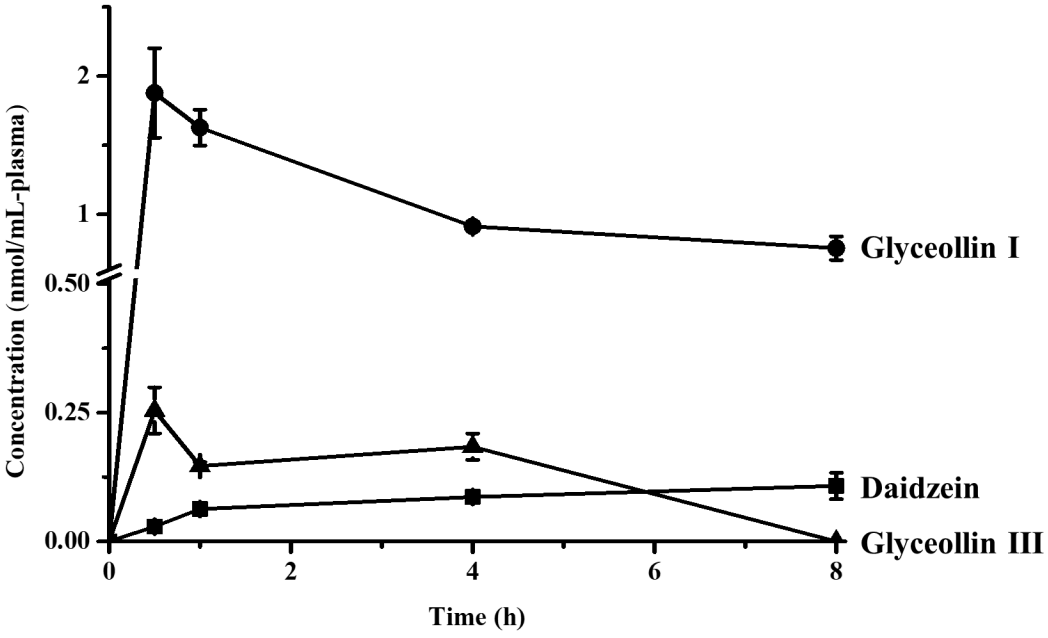


Fig. 2-5 Absorption of total forms of daidzein, glyceollin III, and glyceollin I in the circulatory blood of SD rats.

Deconjugation treatment of plasma sample was performed by β-glucuronidase type B-1 and sulfatase Type H-1 for the detection of total absorption of intact and conjugated forms. Data are expressed as the mean ± S.D. (n = 4).

Table 2-1 Pharmacokinetics of total (intact + conjugates) daidzein, glyceollin III, and glyceollin I in the circulatory blood after single oral administration to SD rats (1.0 mg/kg)

	C_{max} (nmol/mL-plasma)	T_{max} (h)	$T_{1/2}$ (h)	$AUC_{0\text{-}8\ h}$ (nmol•h/mL-plasma)
Daidzein	0.11 ± 0.03	8.0	*n.d.*	0.2 ± 0.1
Equol-OH	0.05 ± 0.02	8.0	*n.d.*	0.2 ± 0.1
Glyceollin III	0.25 ± 0.05	0.5	4.7	1.0 ± 0.2
Glyceollin I	1.9 ± 0.6	0.5	3.1	8.5 ± 0.7

All values are expressed as the mean ± S.D. (n = 4).

C_{max}, maximum plasma concentration.

T_{max} and $T_{1/2}$, plotting test compound levels against the logarithmic concentration; $AUC_{0\text{-}8\ h}$: area under plasma concentration-time curve from 0 to 8 h.

Deconjugation treatment of plasma sample was performed by β-glucuronidase type B-1 and sulfatase Type H-1.

n.d. indicates no determination.

3.2. Transport and metabolism of glyceollins in rat jejunum membrane

The preferable intestinal absorption characteristics of prenylated isoflavones in SD rats compared to their parent isoflavone, daidzein (**Table 2-1**), were examined by *ex vivo* transport experiments of glyceollins using SD rat jejunum membrane. In a 60-min transport experiment with each target (100 μM) in the Ussing chamber, the uptake of intact target in basolateral solution was in the following order of descending preference: glyceollin I (12.5 ± 0.5 pmol/mL) > glyceollin III (10.1 ± 1.4 pmol/mL) > daidzein (2.0 ± 1.0 pmol/mL) (**Fig. 2-6**), as similar to the Caco-2 transport of isoflavones in a hydrophobicity-dependent manner of genistin, daidzin, daidzein, and genistein [86]. The preferable transport of dietary carotenoids was also dependent on their hydrophobicity in Caco-2 cell monolayers [87, 88]. Thus, it seems likely that the higher hydrophobicity (or log *P*) of targets (or penetrants), the more transport (or absorption) would be expected. Apart from the molecular size of daidzein, glyceollin III, and glyceollin I, the close relationship between the uptake and hydrophobicity (or log *P*) of each intact target was in line with the present transport experiments (**Fig. 2-6**) [glyceollin I (uptake, 12.5 ± 0.5 pmol/mL; log *P*, 3.907 ± 0.493); glyceollin III (uptake, 10.1 ± 1.4 pmol/mL; log *P*, 3.60 ± 0.515); daidzein (uptake, 2.0 ± 1.0 pmol/mL; log *P*, 2.632 ± 1.134)].

Basolateral solutions after the *ex vivo* transport experiments of daidzein, glyceollin III, and glyceollin I for 60 min were subjected to LC-TOF/MS analysis to evaluate the preferential transport of conjugated forms. As shown in **Fig. 2-6**, the total uptake of targets (intact and conjugated forms) across the rat jejunum membrane was in a similar descending

order as the uptake of intact targets: glyceollin I (25.5 ± 4.6 pmol/mL), glyceollin III (12.6 ± 1.4 pmol/mL), and daidzein (3.2 ± 1.3 pmol/mL). Of glyceollin I, more than 50% (13.0 ± 4.3 pmol/mL) was conjugated with glucuronide, sulfate, or both (conjugated glyceollin III, 2.4 ± 0.5 pmol/mL). The *ex vivo* transportability of glyceollins and daidzein was in good agreement with their *ex vivo* absorption behavior (**Table 2-1**), indicating that the preferable absorption of glyceollin I may be partly due to its hydrophobicity or high log *P*.

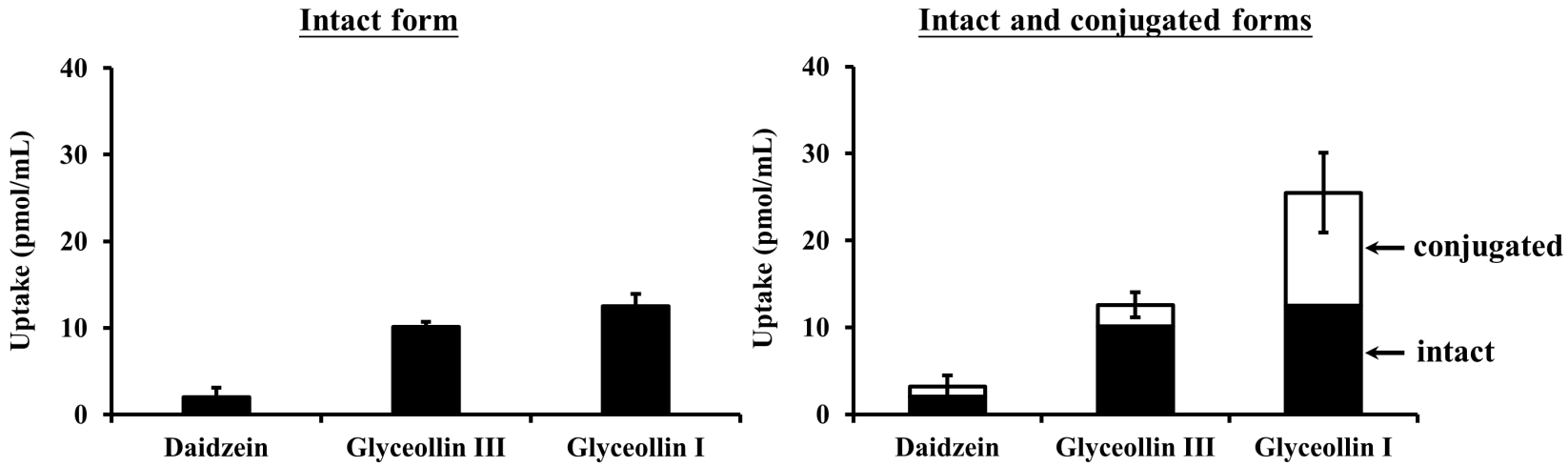


Fig. 2-6 The uptake of intact and a total of intact + conjugated daidzein, glyceollin III, and glyceollin I after 60-min transport experiment using SD rat jejunum membrane.

The black and white columns indicate the uptake of intact and conjugated forms, respectively. All deconjugated samples by enzymatic treatment were analyzed by LC-TOF/MS. Data are expressed as the mean ± S.D. (n = 4).

3.3. Analysis of glyceollin metabolites in rat jejunum membrane by LC-TOF/MS and MALDI-MS

Possible glyceollins metabolites during the *ex vivo* transport experiments in the rat intestinal jejunum membrane were investigated by non-targeting MALDI-MS analysis using the extract of 60-min transported jejunum membrane with the *m/z* range of 100–1000. As shown in **Fig. 2-7**, MALDI-MS peaks corresponding to mono-methylated ([Me-glyceollin III/I – H]$^-$, *m/z* 351.1), mono-sulfated ([Sul-glyceollin III/I – H]$^-$, *m/z* 417.1), and mono-glucuronided forms of glyceollins ([GlcA-glyceollin III/I – H]$^-$, *m/z* 513.1) were successfully detected from the intestinal membrane. The metabolite types of glyceollins were similar to (-)-epigallocatechin (ECG)-phase II metabolism [69]. Since a rapid sulfation reaction in ECG-phase II metabolism was confirmed in rat intestinal membrane, rather than glucuronidation, methylation, and their combination reactions [69], the metabolic speed of glyceollins to sulfated forms would be predominant. However, further study is needed for metabolic preference of glyceollins. For the extract of daidzein-transported membrane, daidzein ([daidzein – H]$^-$, *m/z* 253.1) and hydroxyl equol ([equol-OH – H]$^-$, *m/z* 257.1) were detected, while conjugated metabolites, such as sulfated and glucronidated forms, were not detected, in good agreement with the results in **Fig. 2-7**. The metabolites detected by the MALDI-MS analysis was also confirmed by further LC-TOF/MS analysis (**Fig. 2-8**). More importantly, the broadened and/or two-splitted EIC peaks of glucuronized-glyceollin I ([GlcA-glyceollin I – H]$^-$, *m/z* 513.1402) and

methyled-glyceollin III ([Me-glyceollin III – H]$^-$, *m/z* 351.1238) on LC-TOF/MS chromatograms (**Fig. 2-8**), suggested that at least two isomers with different glucuronized/methylated positions in glyceollin I/III skeleton were produced during the intestinal absorption process of glyceollins, as similar to previous report [89], in which sulfation or glucronidation preferentially occurred at A- and B-rings in flavonoid skeleton. Thus, to identify the isomeric metabolites of glyceollins, further advanced analysis by *e.g.,* tandem MS, is needed.

Although isoflavones were reported to be incorporated via the transcellular and paracellular routes in Caco-2 monolayers [51], glyceollins' transport route(s) in intestinal membrane remains unclear. Inhibitor-aided MALDI-MS imaging technique in previous report [69] would be helpful to clarify the glyceollin incorporation route(s) across the rat intestinal membrane.

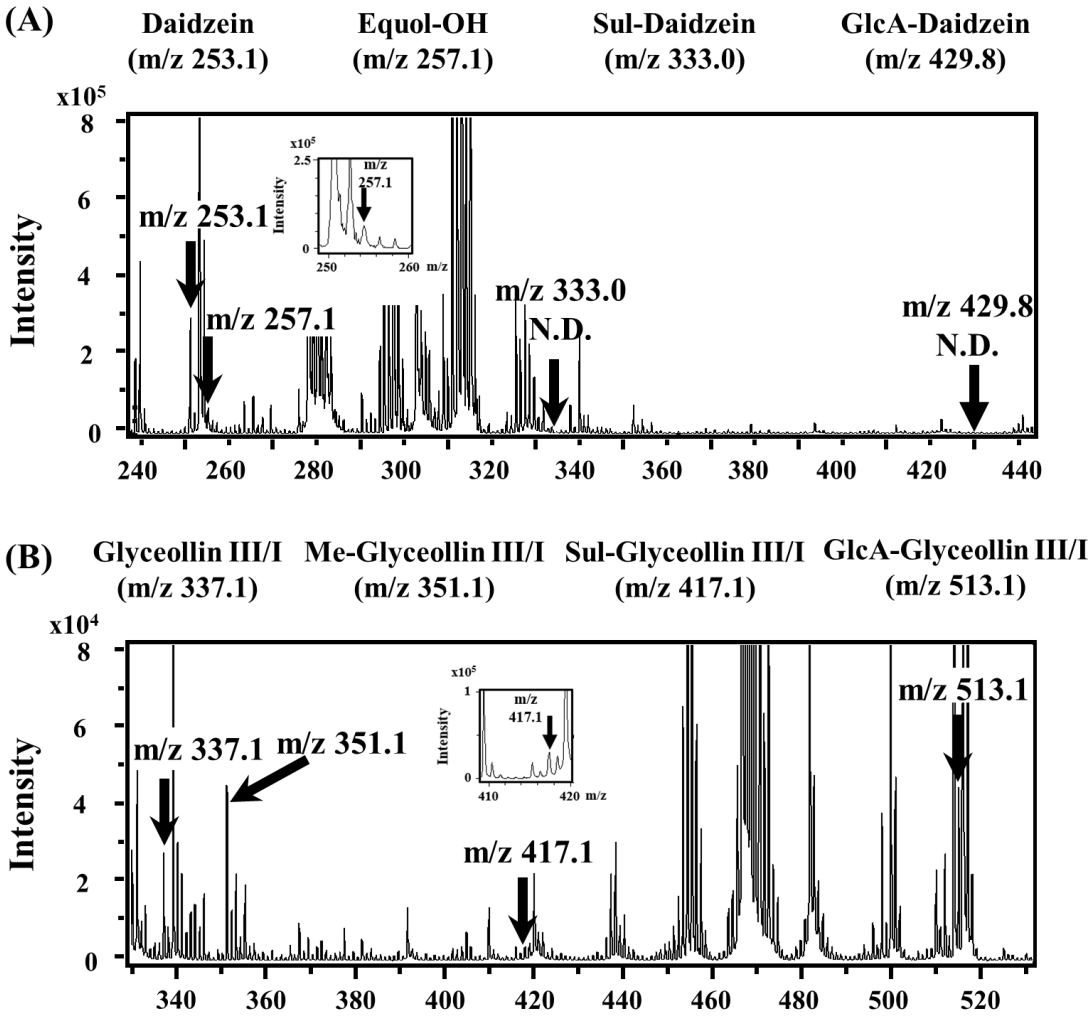


Fig. 2-7 MALDI-MS analysis of the extract of 60-min transported SD rat jejunum membrane.

MALDI-MS spectra show the MS detection patterns of daidzein (A) and glyceollin III/ I (B). Data are expressed as the mean ± S.D. (n = 4). MALDI-MS analysis was performed in negative ion-linear mode using 1,5-DAN as matrix reagent. N.D. indicates no detection. Abbreviations: GlcA (glucuronide), Sul (sulfated), OH (hydroxylated), and Me (methylated).

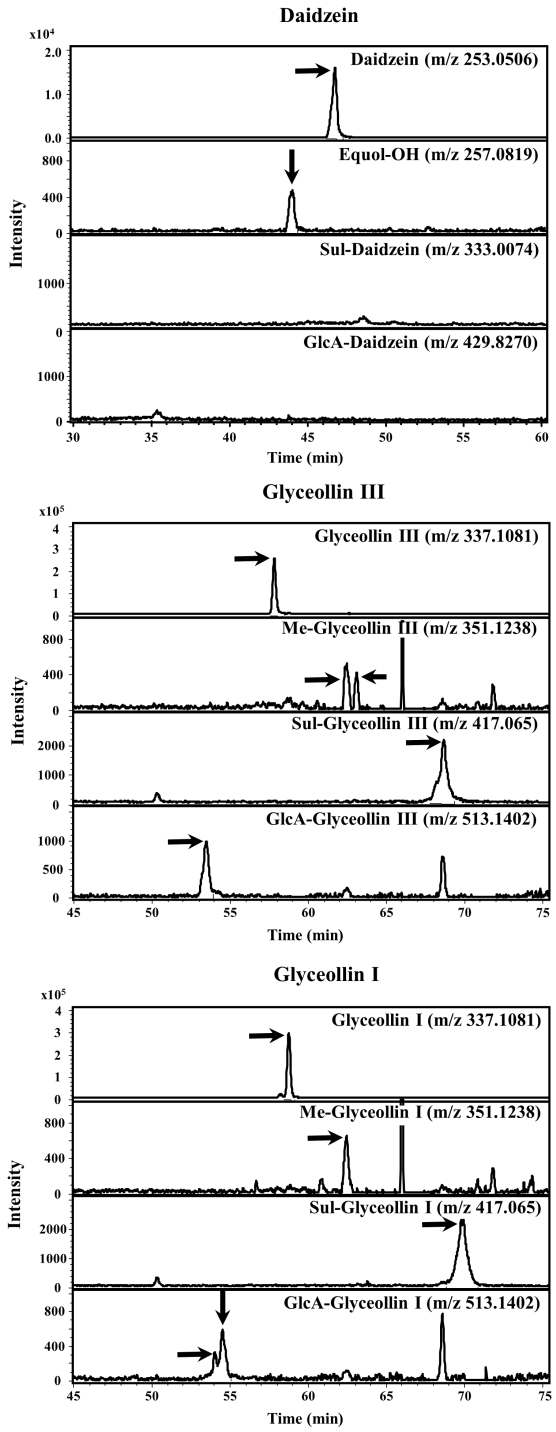


Fig. 2-8 EIC-LC-TOF/MS analysis of metabolites of daidzein and glyceollin III/I in the extract of 60-min transported SD rat jejunum membrane.

LC-TOF/MS analysis was performed in negative ESI mode. Abbreviation: GlcA (glucuronide), Sul (sulfated), OH (hydroxylated), and Me (methylated).

4. Summary

In conclusion, **Chapter II** demonstrates the following findings: (1) upon oral administration of glyceollin III or I (daidzein as control) to SD rats (1.0 mg/kg), no peaks corresponding to their intact forms were detected in plasma by LC-TOF/MS analysis. This indicates that glyceollins III and I, as well as daidzein, were not absorbed into rat circulating bloodstream in their intact forms; (2) enzymatic deconjugation of plasma by β-glucuronidase type B-1 and sulfatase Type H-1 resulted in a successful MS detection of glyceollin III and glyceollin I. This indicates that glyceollins are absorbed in conjugated forms; (3) glyceollins were more absorbable than their parent isoflavone, daidzein, due to their high hydrophobicity. They were metabolized to form sulfated, glucuronized, and methylated conjugates during intestinal absorption process (**Fig. 2-9**). Taken together, prenylation of isoflavones may cause an enhanced intestinal absorption into rat bloodstream in conjugated form.

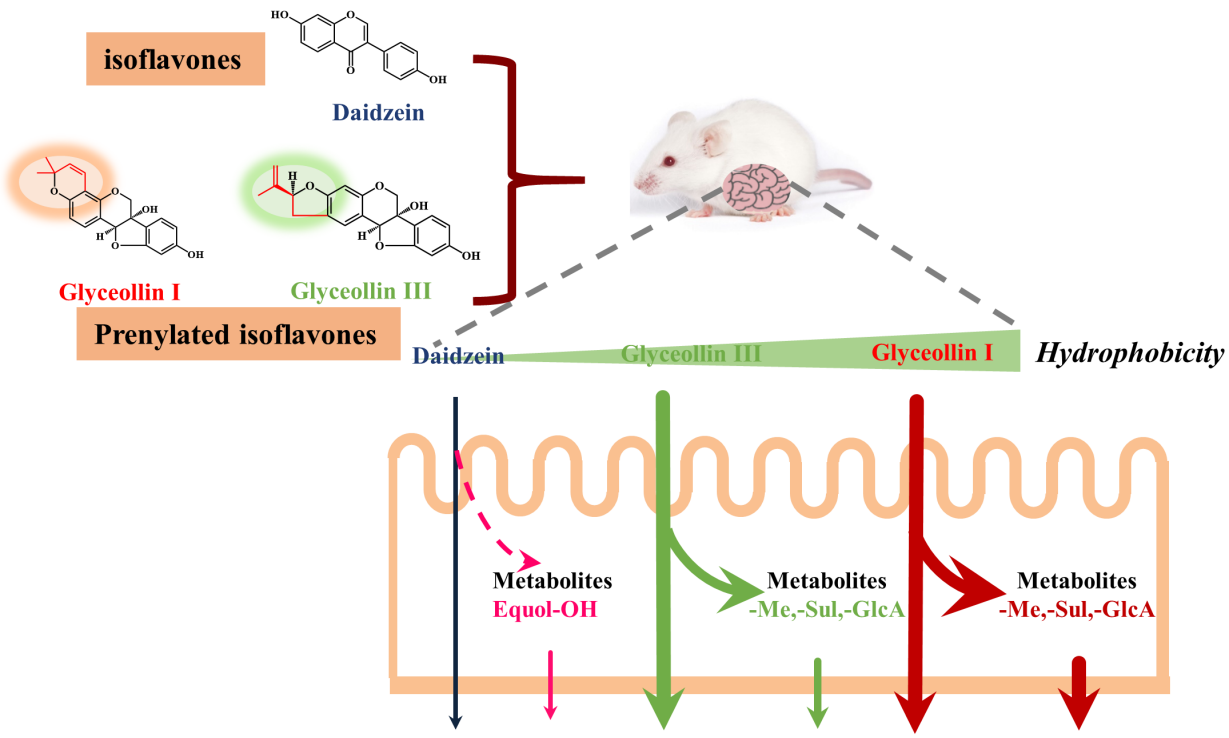


Fig. 2-9 Schematic absorption behavior of daidzein, glyceollin III, and glyceollin I in rat intestinal membrane.

Chapter III

Tissue accumulation of prenylated isoflavones, glyceollins, in Sprague-Dawley rats

1. Introduction

In **Chapter II**, it was demonstrated that glyceollins (prenylated 6α-hydroxy-pterocarpans), caused preferable absorption into the circulatory bloodstream of Sprague-Dawley (SD) rats rather than their parent compound, daidzein. The absorbability of glyceollins was dependent on their hydrophobicity or log *P*. In addition, glyceollins were found to be rapidly (T_{max}, 0.5 h) absorbed and metabolized into conjugated forms, such as methylated, sulfated, and glucuronized conjugates. Along with the preferable absorption characteristics of glyceollins, it was reported that they exerted physiological potentials, such as

improved glucose homeostasis by enhancing hepatic insulin sensitivity, in the liver of diabetic mice after the intake [29]. As summarized in **Table 1-1**, there have been some reports about the preventive effects of prenylated isoflavones, glyceollins, against diverse diseases, such as cardiovascular disease [30], osteoporosis [27], and cancers [16]. Glyceollin I also prevented a recurrent breast cancer by inhibiting the Eleanor RNA cloud and estrogen receptor (ER)α mRNA transcription in the breast cancer cells [17]. These reports strongly suggest that the beneficial roles of glyceollins are physiological event at local organs, but the tissue accumulation after the intake remains unascertained.

It is known that the tissue accumulation of isoflavones (genistein and daidzein), they were detected in intact and/or conjugated forms at the liver of male SD rats [61, 62], as well as in the organ of the placenta [63]. The main conjugates were glucuronized and sulfated forms [63] and they were also reported to play a physiological role through their local accumulation. For example, daidzein and genistein glucuronides inhibited estrogen-dependent proliferation of cancer cells[90] and the hypotensive and vasodilator effects of daidzein sulfates in SHR rats [91]. Thus, in Chapter III, glyceollins III and I, and daidzein (as comparable isoflavone), administered to SD rats by oral to make clear the accumulation dynamics of glyceollins in the circulatory system (the liver, the kidneys, the heart, the lungs, the soleus muscle, and the abdominal aorta). LC-TOF/MS was conducted to identify the intact and conjugated glyceollins in the circulatory system.

2. Materials and methods

2.1. Materials

Glyceollin III and glyceollin I (**Fig. 3-1A**) were purchased from FUJIFILM Wako Pure Chemical Co. (Osaka, Japan). Daidzein was purchased from Tokyo Chemical Ind. Co. (Tokyo, Japan). Sulfatase type H-1 (EC 3.1.6.1, 25 units, from *Helix pomatia*) and β-glucuronidase type B-1 (EC 3.2.1.31, 50 units, from bovine liver) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Hesperetin as an internal standard (IS) was purchased from Sigma-Aldrich. Deionized water prepared using a Milli-Q system (Millipore, Tokyo, Japan) was used for all chemical preparations. For LC-TOF/MS, MS-graded water, MeOH, EtOH, and FA were purchased from Merck (Darmstadt, Germany). All other chemicals were of analytical reagent grade and used without further purification.

2.2. A single oral administration in Sprague-Dawley rats

Administration protocol of glyceollins (1.0 mg/kg) to SD rats was the same as described in **Chapter II**. Three rats at each time-point of 0.5, 1, 3, 6, or 24 h after the administration were sacrificed to obtain blood and target organs. After taking blood from the abdominal aorta, each animal was perfused with 100 mL of phosphate-buffered saline's solution (PBS, pH 7.4, 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4) containing 1 mM PMSF (protease

inhibitor) and 2 mM EDTA-2Na to wash out blood from the circulating system by manual syringe-pumping from the abdominal aorta. Then, target organs (the liver, the kidneys, the lungs, the heart, the soleus muscle, and the abdominal aorta) were collected, immediately frozen in liquid nitrogen and stored at –40°C until LC-TOF/MS analysis. All animal experimental protocols were reviewed and approved by the Animal Care and Use Committee of Kyushu University (permit number: A20-095).

2.3. Preparation of rat organs for LC-TOF/MS analysis

Frozen organs were lyophilized to mash with a BioMasher II (Nippi. Inc., Tokyo, Japan). An aliquot (50 mg) of the dry powder was dissolved in 1.0 mL of 70% EtOH containing 1.0 μM hesperetin (IS, final concentration: 1.0 nmol/mL), following the sonication using a SONIFIRE 250 (Branson Ultrasonics; Emerson Japan Co., Kanagawa, Japan) with an output control of 3 for 10 s thrice at 4°C. Then the sample solution was homogenized with a Polytron homogenizer (Kinmatica AG; Luzern, Switzerland) at 20,000 rpm for 30 s twice. After centrifugation at 14,000 × *g* for 15 min at 25°C, the obtained supernatant was ultra-filtered using an Amicon Ultra 0.5-mL 3K centrifugal filter (Millipore, Carrigtwohill, Ireland) at 14,000 × *g* for 30 min at 25°C. The filtrate was evaporated to dryness.

For quantitative assay of daidzein, glyceollin III, and glyceollin I in the organs of SD rat, the aforementioned dried filtrate was dissolved in 100 μL of 100 mM sodium acetate buffer (pH 4.0) and was subjected to LC-TOF/MS analysis for determining either intact or conjugated metabolites. For the assay of a total of intact and conjugated forms, enzymatic deconjugation treatments were performed with sulfatase type H-1 (25 units, 20 μL) and β-glucuronidase type B-1 (50 units, 10 μL). Namely, both enzyme solutions were added to the above sample solution (100 μL), following 12 h-incubation at 37°C. Sample solutions with or without enzymatic treatment were mixed with 100 μL of EtOH, and then centrifuged at 14,000 × *g* for 15 min at 25°C. The supernatant was evaporated to dryness. Prior to LC-TOF/MS analysis, the sample was dissolved in 50 μL of 50% EtOH and filtrated with a 0.45-μm Millex-LH Syringe Driven Filter Unit (Millex, Tokyo, Japan). Glyceollin assay in rat plasma was the same as the procedures in **Chapter II**.

2.4. LC-TOF/MS analysis

The conditions for LC separation and MS analysis were the same as **Chapter II**. Targets in the organs were quantified with the aid of hesperetin as IS. Thus, daidzein ($[M - H]^-$: 253.0506 *m/z*), and glyceollins III and I (each $[M - H]^-$: 337.1081 *m/z*) in the organs were quantified using calibration curves of y = 0.4103x + 0.026 [0.05–1.0 nmol/mL; r^2 = 0.9913; limit of detection (LOD),

3.0 pmol/mL] for daidzein, y = 0.3354x – 0.0242 (0.0625–1.0 nmol/mL; r^2 = 0.9809; LOD, 1.0 pmol/mL) for glyceollin III, and y = 0.681x – 0.0338 (0.0625–1.0 nmol/mL; r^2 = 0.9954; LOD, 0.9 pmol/mL) for glyceollin I, where y is the ratio of observed peak area against that of the IS and x is the concentration (nmol/mL). The amount of target (nmol/mL) obtained from 50 mg dry tissue sample was converted to nmol/g tissue.

2.5. Statistical analysis

Pharmacokinetic analysis of concentration-time data was performed using a GraphPad Prism software (GraphPad; La Jolla, CA, USA). The area under the curve up to 6 h ($AUC_{0\text{-}6\ h}$) were obtained directly from the different tissue concentration-time plots. Results are expressed as the mean ± standard deviation (S.D.).

3. Results and discussion

3.1. Tissue accumulation of glyceollins in rat organs after oral administration

Oral administration experiments to SD rats at a dose of 1.0 mg/kg with daidzein, glyceollin III, and glyceollin I were conducted to investigate the

accumulation in organs. As shown in **Fig. 3-1**, it was apparent that intact forms of both glyceollin III and glyceollin I were detected in targeted organs at 0.5 h after the administration, whereas intact form of daidzein was detected only in the liver and the kidney. This strongly indicates that both the prenylated isoflavones were rapidly and preferably distributed and accumulated into circulatory organs after entering the bloodstream, as compared to the parent isoflavone, daidzein. **Fig. 3-1** also revealed that the sulfated and/or glucuronized forms of glyceollins were predominant in the accumulated organs (the liver, the kidneys, the heart, the lungs, the soleus muscle, and the abdominal aorta). Urpi-Sarda *et al*.[64] reported that a daily intake of genistein (7.6 mg/kg) or daidzein (2.9 mg/kg) to the ewes for 1 month resulted in an apparent tissue accumulation of intact and conjugated isoflavones at the liver, the kidneys, the aorta, the lungs, the heart, and the muscle, as similar to the accumulation behavior of glyceollins (**Fig. 3-1**); at the kidney the accumulation of daily administered daidzein was reached to 71.23 nmol/g-tissue. Similarly, in this study, daidzein and hydroxyl equol (equol-OH) ($[M - H]^-$: 257.0819 *m/z*), a typical metabolite of daidzein [58], were detected in conjugated form in the liver and the kidneys, whereas a significant accumulation of equol-OH as well as daidzein were not detected in the other organs in the present LC-MS and dosage (1.0 mg/kg) conditions (**Fig. 3-1**). As shown in **Fig. 3-1 (**T= 3 and 6 h**)** for glyceollin accumulation, glyceollin I was detected in all organs as conjugated form as long as 6 h after the administration, while a rapid disappearance of accumulated glyceollin III was observed in the

organs, except for the liver and the kidneys at > 3 h after the administration. At 24 h after the administration, glyceollins and daidzein (and equol-OH) were completely disappeared from all the organs of 1.0 mg/kg-loaded SD rats (**Fig. 3-1,** T=24 h). This strongly indicates that the prenyl moiety in glyceollins might affect accumulation and retention into circulatory organs after entering the bloodstream. As summarized in **Table 3-1**, conjugated glyceollin III and glyceollin I were predominantly accumulated in the liver and the kidneys at any time-points up to 6 h, whereas in the heart, the lungs, the soleus muscle, and the abdominal aorta the accumulation of amounts conjugates were almost equal to those of intact forms [*e.g.,* heart: intact glyceollin I at 3 h, 0.16 nmol/g-dry tissue; conjugated one, 0.30 nmol/g-dry tissue (0.46 nmol/g-dry tissue minus 0.16 nmol/g-dry tissue); soleus muscle: intact glyceollin III at 1 h, 0.15 nmol/g-dry tissue; conjugated one, 0.01 nmol/g-dry tissue]. This strongly indicates that the accumulation potencies of the intact and conjugated forms of glyceollins in circulatory organs, except for the liver and the kidneys, may be comparable.

The amount of tissue accumulation of other phytochemicals, such as ferulic acid [40, 41], caffeic acid [42, 43], naringin [44], and daidzein [45, 46], in organs (the liver, the kidneys, the lungs, and the heart) have summarized in **Table 3-2**. They can be distributed and accumulated in the organs rapidly (< 1 h) in their intact and/or conjugated forms except for soleus muscle and abdominal aorta, as similar to the present results of glyceollins. The accumulation of ferulic acid [41], caffeic acid [43], naringin [44], and daidzein [46] in different organs preferably

in the following order: the kidney > the liver > the lung > the heart (**Table 3-2**). The liver would be the predominant accumulation organ for glyceollins. However, further study is needed for the accumulation preference of glyceollins. In this study, it was apparent that the accumulation potency of glyceollins in intact and total form were much higher than the phytochemicals in **Table 3-2**. For intact accumulation, approximate C_{max} at the liver: ferulic acid at 3.3 mg/kg, 1.62 nmol/g-tissue [41]; caffeic acid at 3.01 mg/kg, 1.93 nmol/g-tissue [43]; glyceollin I at 1.0 mg/kg, 1.4 nmol/g-tissue; glyceollin III, 1.7 nmol/g-tissue (**Table 3-1**). For total accumulation, naringin, 42 mg/kg [44] was reported to be showing a C_{max} of 78.7 nmol/g-dry tissue in the liver. While the efficiency of glyceollins per dose (C_{max}/dose of glyceollin I, 42.3 nmol/g-dry tissue/mg-dose; glyceollin III, 13.0 nmol/g-dry tissue/mg-dose) was predominant rather than that of naringin (C_{max}/dose: 1.87 nmol/g-drytissue/mg-dose). Similarly, C_{max}/dose of total daidzein (daidzein plus its metabolite equol-OH), 0.37 nmol/g-dry tissue/mg-dose for the liver was also higher than that of 0.26 nmol/g-dry tissue/mg-dose for daidzein [46] (**Table 3-2)**. It has been reported that the prenylation may enhance tissue accumulation by increasing hydrophobicity or log P [92], as similar to enhanced absorbability of glyceollins into the bloodstream (**Chapter II)**. A good example was a 10-times higher accumulation of 8-prenyl naringenin in the mouse gastrocnemius muscle than that of naringenin [34]. Prenylation of quercetin (8-prenyl quercetin) also caused a higher tissue accumulation in the liver and the kidney [35]. Thus, it was

concluded that prenylation of phytochemicals may be effective for promoting absorption and accumulation, according to previous [34, 35] and the present results (**Chapter II** and **Fig. 3-1).**

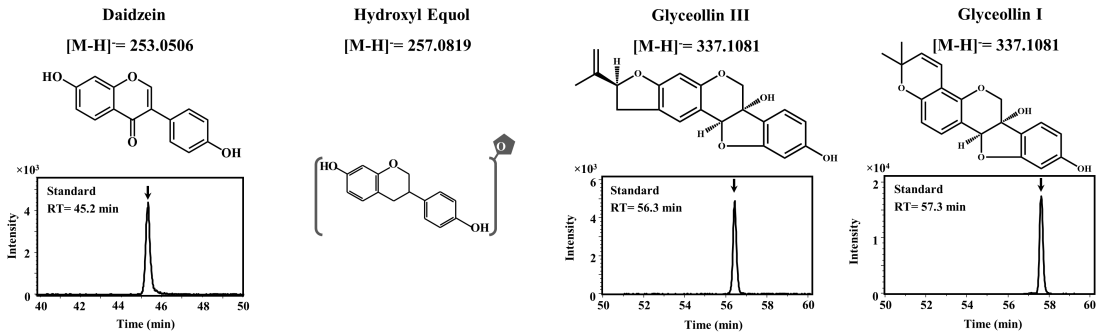


Fig. 3-1 Tissue Distribution of daidzein, glyceollin III, and glyceollin I in the plasmas and organs of SD rats after single oral administration.

Oral administration of glyceollins was performed at a dose of 1.0 mg/kg to SD rats. At 0.5, 1, 3, 6, and 24 h after the administration, organs (the liver, the kidneys, the heart, the lungs, the soleus muscle, the abdominal aorta) were taken from the rats for LC-TOF/MS analysis. The concentration of each standard was 1 μM. MS chromatograms show the elution of each target in plasma and organs at T = 0.5, 1, 3, 6, and 24 h. Intact or Intact + Conjugates indicate without or with enzymatic treatment by β-glucuronidase type B-1 and sulfatase type H-1 treatments. N.D. indicates no detection. Trace indicates that the amounts is less than 3-times of LOD (LOD: 3.0, 1.0, and 0.9 pmol/mL for daidzein, glyceollin III and glyceollin I, respectively).

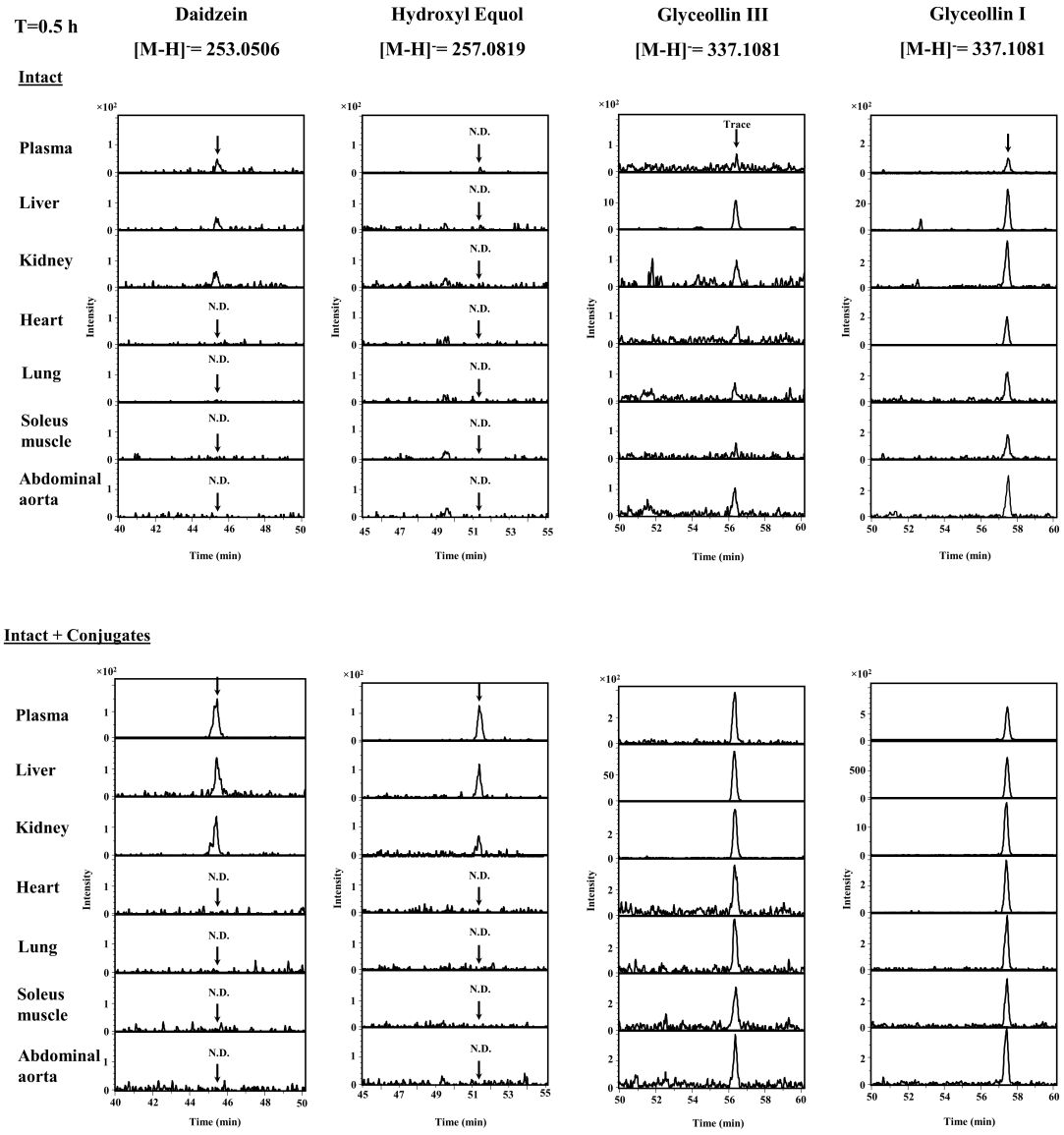


Fig. 3-1 (Continued)

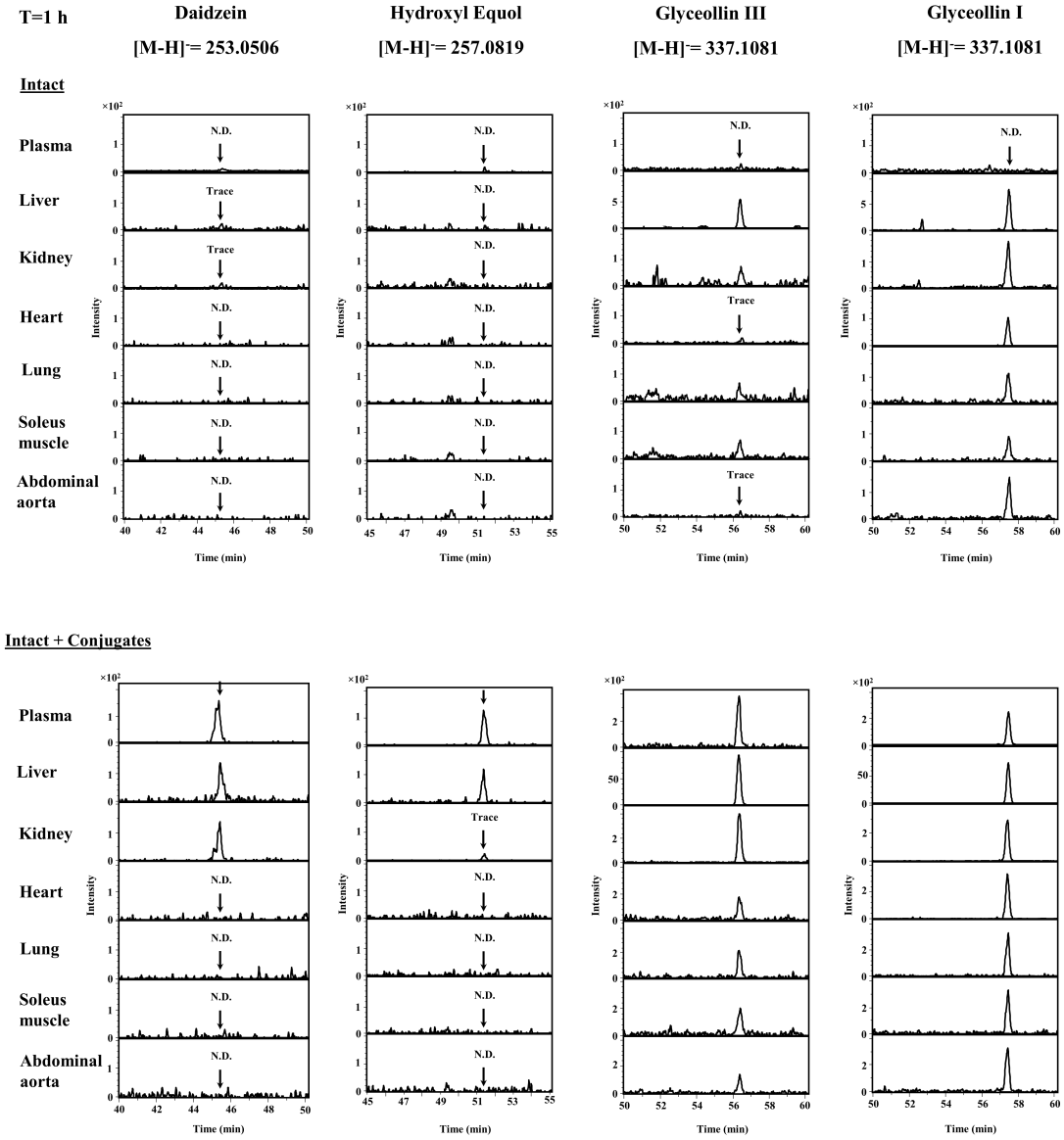


Fig. 3-1 (Continued)

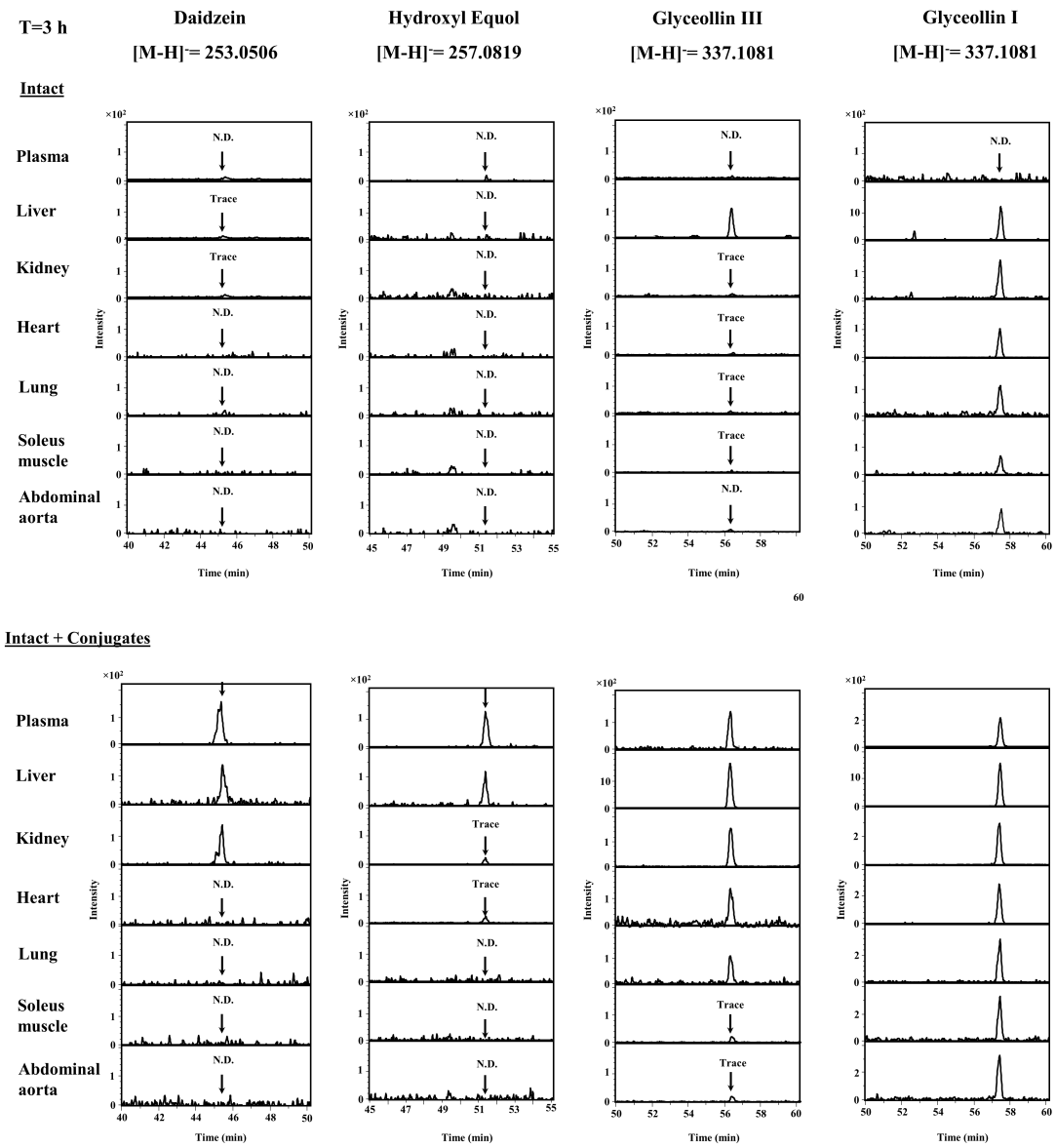


Fig. 3-1 (Continued)

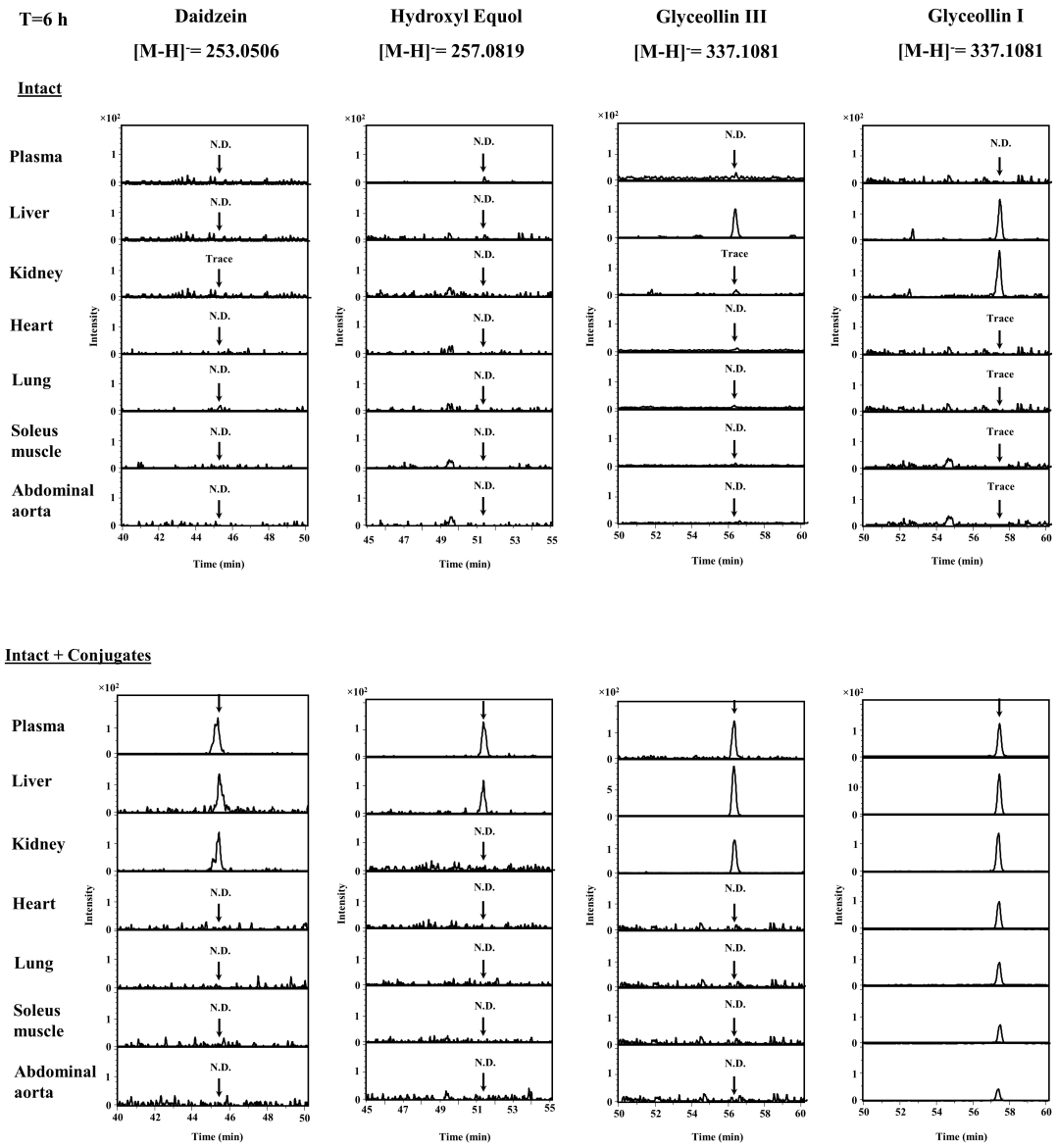


Fig. 3-1 (Continued)

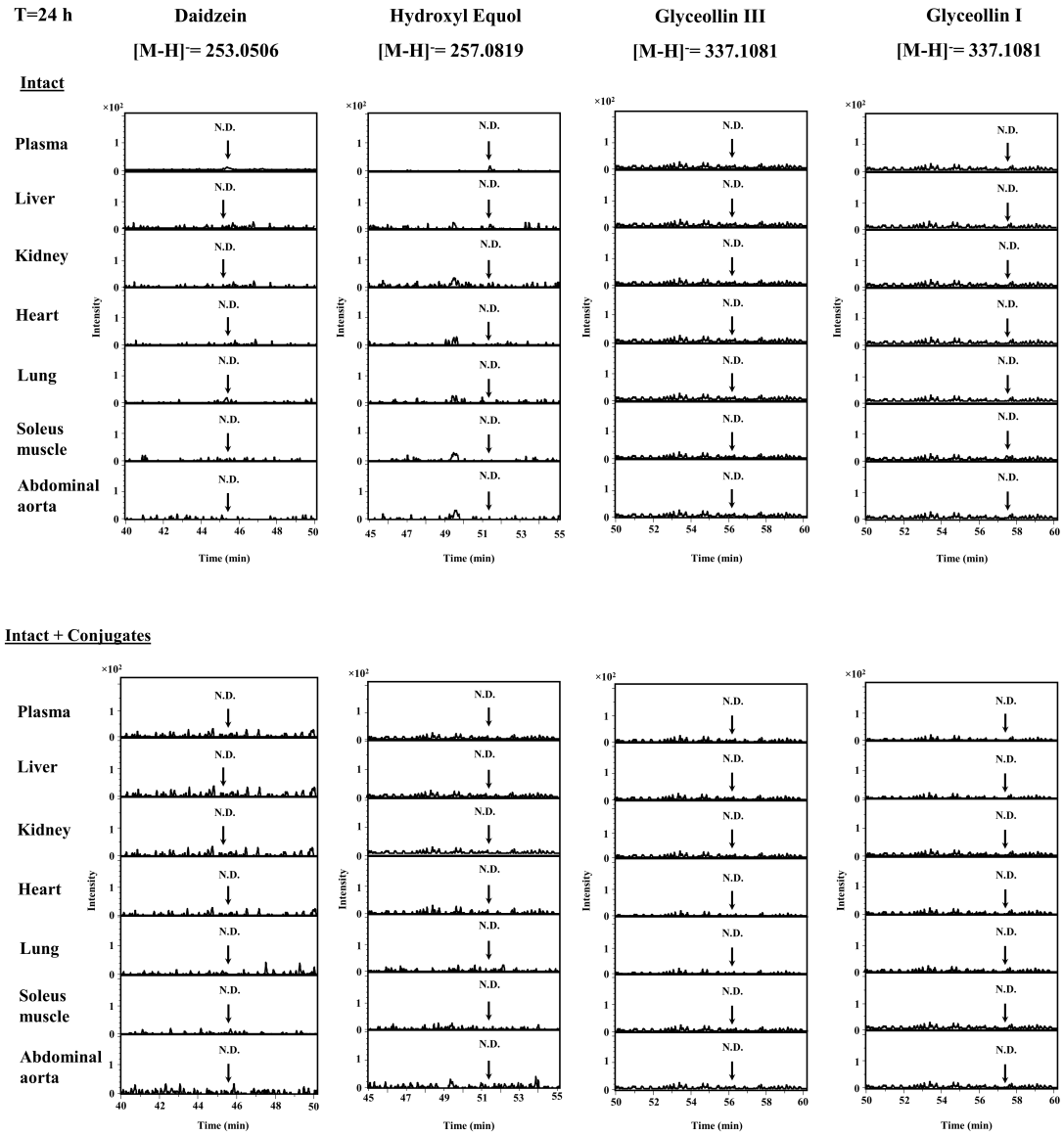


Fig. 3-1 (Continued)

Table 3-1. Tissue concentrations of daidzein, glyceollin III, and glyceollin I after single oral administration in SD rats at a dose of 1.0 mg/kg body weight [a]

Organ	Time	Intact [b]				Total (Intact + Conjugates) [b]			
	(h)	daidzein	equol-OH	glyceollin III	glyceollin I	daidzein	equol-OH	glyceollin III	glyceollin I
plasma (nmol/mL-plasma)	0.5	0.01 ± 0.01	*n.d.*[c]	*trace*[d]	0.17 ± 0.01	0.08 ± 0.02	0.03 ± 0.01	0.19 ± 0.05	0.97 ± 0.06
	1	*n.d.*	*n.d.*	*n.d.*	*n.d.*	0.04 ± 0.02	0.01 ± 0.01	0.14 ± 0.01	0.62 ± 0.03
	3	*n.d.*	*n.d.*	*n.d.*	*n.d.*	0.05 ± 0.02	0.01 ± 0.01	0.13 ± 0.01	0.44 ± 0.01
	6	*n.d.*	*n.d.*	*n.d.*	*n.d.*	0.08 ± 0.01	0.01 ± 0.01	0.14 ± 0.02	0.38 ± 0.01
	24	*n.d.*	*n.d.*	*n.d.*	*n.d.*	*n.d.*	*n.d.*	*n.d.*	*n.d.*
liver (nmol/g-dry tissue)	0.5	0.01 ± 0.01	*n.d.*	1.7 ± 0.3	1.4 ± 0.2	0.08 ± 0.04	0.23 ± 0.06	13.0 ± 2.1	42.3 ± 4.9
	1	*trace*	*n.d.*	0.60 ± 0.10	0.75 ± 0.17	0.02 ± 0.02	0.08 ± 0.02	8.6 ± 1.0	25.6 ± 3.4
	3	*trace*	*n.d.*	0.35 ± 0.05	0.50 ± 0.10	0.03 ± 0.03	*trace*	4.0 ± 0.9	17.0 ± 4.4
	6	0.01 ± 0.01	*n.d.*	0.14 ± 0.08	0.29 ± 0.04	0.14 ± 0.06	0.02 ± 0.02	3.4 ± 0.7	9.8 ± 1.5
	24	*n.d.*	*n.d.*	*n.d.*	*n.d.*	*n.d.*	*n.d.*	*n.d.*	*n.d.*
kidney (nmol/g-dry tissue)	0.5	0.02 ± 0.01	*n.d.*	0.34 ± 0.10	0.39 ± 0.05	0.21 ± 0.04	0.03 ± 0.03	1.6 ± 0.5	2.3 ± 0.4
	1	*trace*	*n.d.*	0.24 ± 0.03	0.28 ± 0.01	0.20 ± 0.02	*trace*	1.1 ± 0.2	1.5 ± 0.4
	3	*trace*	*n.d.*	*trace*	0.18 ± 0.01	0.09 ± 0.06	*trace*	0.60 ± 0.10	1.0 ± 0.03
	6	*trace*	*n.d.*	*trace*	0.14 ± 0.08	0.18 ± 0.07	*n.d.*	0.70 ± 0.10	0.60 ± 0.10
	24	*n.d.*	*n.d.*	*n.d.*	*n.d.*	*n.d.*	*n.d.*	*n.d.*	*n.d.*
heart (nmol/g-dry tissue)	0.5	*n.d.*	*n.d.*	0.26 ± 0.11	0.41 ± 0.02	*n.d.*	*n.d.*	0.53 ± 0.06	0.88 ± 0.09
	1	*n.d.*	*n.d.*	*trace*	0.20 ± 0.06	*n.d.*	*n.d.*	0.27 ± 0.11	0.70 ± 0.20
	3	*n.d.*	*n.d.*	*trace*	0.16 ± 0.01	*n.d.*	*n.d.*	0.21 ± 0.11	0.46 ± 0.07
	6	*n.d.*	*n.d.*	*n.d.*	*trace*	*n.d.*	*n.d.*	*n.d.*	0.31 ± 0.03
	24	*n.d.*	*n.d.*	*n.d.*	*n.d.*	*n.d.*	*n.d.*	*n.d.*	*n.d.*
lung (nmol/g-dry tissue)	0.5	*n.d.*	*n.d.*	0.26 ± 0.05	0.30 ± 0.11	*n.d.*	*n.d.*	0.44 ± 0.04	0.50 ± 0.10
	1	*n.d.*	*n.d.*	0.18 ± 0.05	0.26 ± 0.04	*n.d.*	*n.d.*	0.39 ± 0.13	0.61 ± 0.08
	3	*n.d.*	*n.d.*	*trace*	0.19 ± 0.04	*n.d.*	*n.d.*	0.20 ± 0.08	0.40 ± 0.09
	6	*n.d.*	*n.d.*	*n.d.*	*trace*	*n.d.*	*n.d.*	*n.d.*	0.20 ± 0.03
	24	*n.d.*	*n.d.*	*n.d.*	*n.d.*	*n.d.*	*n.d.*	*n.d.*	*n.d.*
soleus muscle (nmol/g-dry tissue)	0.5	*n.d.*	*n.d.*	0.16 ± 0.03	0.22 ± 0.04	*n.d.*	*n.d.*	0.21 ± 0.07	0.37 ± 0.05
	1	*n.d.*	*n.d.*	0.15 ± 0.01	0.17 ± 0.03	*n.d.*	*n.d.*	0.16 ± 0.02	0.34 ± 0.06
	3	*n.d.*	*n.d.*	*trace*	0.14 ± 0.02	*n.d.*	*n.d.*	*trace*	0.24 ± 0.05
	6	*n.d.*	*n.d.*	*n.d.*	*trace*	*n.d.*	*n.d.*	*n.d.*	0.20 ± 0.03
	24	*n.d.*	*n.d.*	*n.d.*	*n.d.*	*n.d.*	*n.d.*	*n.d.*	*n.d.*

abdominal aorta (nmol/g-dry tissue)	0.5	*n.d.*	*n.d.*	0.26 ± 0.06	0.43 ± 0.03	*n.d.*	*n.d.*	0.41 ± 0.17	0.50 ± 0.06
	1	*n.d.*	*n.d.*	*trace*	0.42 ± 0.01	*n.d.*	*n.d.*	0.30 ± 0.14	0.43 ± 0.12
	3	*n.d.*	*n.d.*	*n.d.*	0.10 ± 0.03	*n.d.*	*n.d.*	*trace*	0.21 ± 0.10
	6	*n.d.*	*n.d.*	*n.d.*	*n.d.*	*n.d.*	*n.d.*	*n.d.*	0.13 ± 0.02
	24	*n.d.*	*n.d.*	*n.d.*	*n.d.*	*n.d.*	*n.d.*	*n.d.*	*n.d.*

[a] All values are expressed as the mean ± S.D. (n = 3).

[b] Intact/Total: without/with enzymatic treatments by β-glucuronidase type B-1 and sulfatase type H-1.

[c] *n.d.* indicates no determination.

[d] *Trace*: < 3, < 1, and < 0.9 pmol/mL for daidzein, glyceollin III, and glyceollin I, respectively.

Table 3-2. Pharmacokinetics of phytochemicals in blood and organs after a single oral administration in SD rats in literature

compound	dose (mg/kg)	C_{max} (nmol/g-tissue) (intact + conjugates)							reference
		plasma (μmol/mL or nmol/mL)	liver	kidney	heart	lung	soleus muscle	abdominal aorta	
daidzein (intact)	1.0	0.08 (0.5 h)[a]	0.14 (0.5 h)	0.21 (0.5 h)	*n.a.*[b]	*n.a.*	*n.a.*	*n.a.*	this study
equol-OH	daidzein dosage	0.03 (0.5 h)	0.23 (0.5 h)	0.03 (0.5)	*n.a.*	*n.a.*	*n.a.*	*n.a.*	this study
glyceollin III (total[c])	1.0	0.19 (0.5 h)	12.9 (0.5 h)	1.6 (0.5 h)	0.53 (0.5 h)	0.44 (0.5 h)	0.21 (0.5 h)	0.41 (0.5 h)	this study
glyceollin I (total)	1.0	0.97 (0.5 h)	42.3 (0.5 h)	2.3 (0.5 h)	0.88 (0.5 h)	0.61 (1.0 h)	0.37 (0.5 h)	0.50 (0.5 h)	this study
ferulic acid (intact)	3.3	5.73 (0.66 h)	1.62 (*n.a.*)	12.66 (*n.a.*)	0.21 (*n.a.*)	0.19 (*n.a.*)	*n.a.*	*n.a.*	[40, 41]
caffeic acid (intact)	3.01	*n.a.*	1.93 (0.22 h)	1.85 (0.25 h)	1.48 (0.25 h)	1.67 (0.25 h)	*n.a.*	*n.a.*	[42, 43]

naringin (intact)	42	0.309 (0.5 h)	12.2 (0.25 h)	13.2 (0.25 h)	0.36 (0.25 h)	1.88 (1 h)	0.62 (0.25 h)	*n.a.*	[44]
naringin (total)	42	12.9 (0.25 h)	78.7 (6 h)	46.7 (6 h)	5.24 (6 h)	9.32 (1 h)	1.07 (6 h)	*n.a.*	[44]
daidzein (total)	1.64	0.38 (0.89 h)	0.43 (*n.a.*)	0.25 (*n.a.*)	0.02 (*n.a.*)	0.18 (*n.a.*)	*n.a.*	*n.a.*	[45, 46]

[a] Parentheses indicate the T_{max} at each organ.

[b] *n.a.* indicates no available.

[c] Total indicates the accumulation of both intact and conjugated forms.

3.2. Pharmacokinetics of glyceollin accumulation in rat organs after oral administration

Table 3-3 summarizes the pharmacokinetics of total (intact + conjugated) glyceollins accumulated in the organs of SD rat at a dose of 1.0 mg/kg. It was demonstrated that glyceollins as well as their parent daidzein were rapidly (T_{max}, 0.5 h) distributed into the organs after the administration. Considering that the T_{max} (0.5 h) of glyceollins into the circulatory bloodstream was the same as T_{max} of accumulation (**Table 3-2**), their tissue distribution may occur subsequently, as they enter the circulatory bloodstream. Glyceollin I as well as glyceollin III was preferably accumulated in the organs, in the same order of the liver > the kidneys > the heart > the lungs > the soleus muscle, the abdominal aorta. In contrast, daidzein and its predominant metabolite equol-OH were detected only in the liver and the kidneys at less than 1/100-times lower amounts than those of glyceollins, though accumulation of daidzein and equol-OH into other organs cannot be ruled out. In addition, considering that although *e.g.,* $AUC_{0\text{-}6\ h}$ in plasma for glyceollin III (0.73 ± 0.05 nmol·h/mL) was twice than that of daidzein (0.34 ± 0.05 nmol·h/mL), the $AUC_{0\text{-}6\ h}$ in the liver for glyceollin III (32.5 ± 2.9 nmol·h/g-dry tissue) was 100-times higher than that of daidzein (0.32 ± 0.05 nmol·h/g-dry tissue) (**Table 3-3**), distribution of glyceollins towards organs may be much preferential rather than their parent isoflavone daidzein. Besides, the $AUC_{0\text{-}6\ h}$ data regarding a total (intact and conjugated) forms of glyceollins, suggested that glyceollin I may preferably (at least 1.3-times higher) accumulate

into organs rather than glyceollin III [*e.g.,* $AUC_{0\text{-}6\ h}$ in the kidney: glyceollin I, 6.4 ± 2.1 nmol·h/g-dry tissue; glyceollin III, 4.9 ± 0.6 nmol·h/g-dry tissue, 6.4/4.9=1.3; in other organs: 113.2/32.5=3.5 (liver); 2.8/1.1=2.5 (heart); 2.5/1.2=2.1(lung); 1.5/0.3=5 (soleus muscle); 1.3/0.5=2.6 (abdominal aorta)] (**Table 3-3**). According to the pharmacokinetics of glyceollins (**Table 3-3**), absorption ratio of glyceollin I by accounting for total amounts of plasma absorption and tissue accumulation (except for the soleus muscle and the abdominal aorta) was estimated to be > 13.4% when 1.0 mg/kg of glyceollin I (4.2% of glyceollin III) was administered to SD rat, being 10-times more absorbable than daidzein and its metabolite equol-OH (0.35% of daidzein + equol-OH). Comparing the total accumulation amounts in organs with those in plasma, 89.3% [= 9.2 (10.3-1.1) μg/10.3 μg × 100] glyceollin III and 83.9% [= 28.2 (33.6-5.4) μg/33.6 μg × 100] glyceollin I were preferentially distributed into organs (**Table 3-3**), which highlights the significantly preferential accumulation of glyceollins compared to daidzein [24.5%, 0.13 (0.53-0.40) μg/0.53 μg × 100] and its metabolite equol-OH [44.4%, 0.16 (0.36-0.20) μg/0.36 μg × 100]. The high absorbability of 13.4% of glyceollin I was comparable to drug of nefazodone (15%) [93]. In addition, Kida *et al.* reported the 3.4% absorbability of EGCG when it was loaded to Wistar male rat (100 mg/kg) [94]. Taken together, glyceollin I is a phytochemical having high bioavailability leading to a high efficiency to maintain health at lower intake.

Table 3-3. Absorbability of total (intact + conjugates) daidzein, glyceollin III, and glyceollin I in organs after a single oral administration in SD rats at a dose of 1.0 mg/kg body weight [a]

		Total weight (mL or mg)	$AUC_{0\text{-}6\ h}$ (nmol•h/mL-plasma or g- tissue)	Total accumulation amount in organ (nmol)	Total accumulation amount in organ (μg)	Absorption ratio (%)
daidzein	plasma	16.25 ± 1.42	0.34 ± 0.05	1.3 ± 0.3 [c]	0.40 ± 0.10	
	liver	1.74 ± 0.08	0.32 ± 0.05	0.15 ± 0.08	0.05 ± 0.03	
	kidney	1.13 ± 0.04	1.0 ± 0.2	0.24 ± 0.09	0.08 ± 0.03	
	heart	0.59 ± 0.05	*n.d.*	*n.d.*	*n.d.*	
	lung	0.39 ± 0.05	*n.d.*	*n.d.*	*n.d.*	
	soleus muscle	–	*n.d.*	– [d]	–	
	abdominal aorta	–	*n.d.*	–	–	
	sum			1.7 ± 0.6	0.53 ± 0.05	0.21 ± 0.01
equol-OH	plasma	16.25 ± 1.42	0.07 ± 0.01	0.59 ± 0.25	0.20 ± 0.08	
	liver	1.74 ± 0.08	0.17 ± 0.06	0.40 ± 0.10	0.15 ± 0.04	
	kidney	1.13 ± 0.04	0.01 ± 0.01	0.03 ± 0.01	0.01 ± 0.005	
	heart	0.59 ± 0.05	*n.d.*	*n.d.*	*n.d.*	
	lung	0.39 ± 0.05	*n.d.*	*n.d.*	*n.d.*	
	soleus muscle	–	*n.d.*	–	–	
	abdominal aorta	–	*n.d.*	–	–	
	sum			1.0 ± 0.3	0.36 ± 0.09	0.14 ± 0.02

glyceollin III	plasma	17.29 ± 1.50	0.73 ± 0.05	3.3 ± 0.3	1.1 ± 0.1	
	liver	1.98 ± 0.50	32.5 ± 2.9	24.8 ± 4.1	8.4 ± 1.4	
	kidney	1.18 ± 0.05	4.9 ± 0.6	1.9 ± 0.8	0.60 ± 0.20	
	heart	0.49 ± 0.04	1.1 ± 0.3	0.26 ± 0.03	0.09 ± 0.01	
	lung	0.38 ± 0.04	1.2 ± 0.3	0.17 ± 0.01	0.06 ± 0.005	
	soleus muscle	–	0.30 ± 0.07	–	–	
	abdominal aorta	–	0.50 ± 0.10	–	–	
	sum			30.4 ± 4.5	10.3 ± 1.5	4.2 ± 0.6
glyceollin I	plasma	16.45 ± 0.85	2.8 ± 0.1	16.0 ± 0.8	5.4 ± 0.3	
	liver	1.86 ± 0.40	113.2 ± 17.6	79.3 ± 9.3	26.8 ± 3.1	
	kidney	1.35 ± 0.08	6.4 ± 2.1	3.1 ± 0.6	1.1 ± 0.2	
	heart	0.67 ± 0.05	2.8 ± 0.3	0.61 ± 0.06	0.23 ± 0.05	
	lung	0.39 ± 0.05	2.5 ± 0.4	0.24 ± 0.05	0.08 ± 0.02	
	soleus muscle	–	1.5 ± 0.2	–	–	
	abdominal aorta	–	1.3 ± 0.4	–	–	
	sum			99.2 ± 9.9	33.6 ± 3.4	13.4 ± 0.8

[a] All values are the sum of intact and conjugated forms, which are expressed as the mean ± S.D. (n = 3).

[b] *n.d.*: not determined.

[c] Amount of targets in plasma was calculated from a total plasma, according to the estimation by Bijsterbosch *et al* [95]: plasma volume (mL) = 0.026 × body weight (g) + 0.9.

[d] –: not measured

3.3. Accumulation of glyceollin metabolites in rat organs

According to the finding in **Chapter II** that glyceollins were metabolized to form sulfated and/or glucuronized conjugates in blood during intestinal absorption process, accumulation of the two possible conjugates was then investigated in this study [96, 97]. As shown in **Fig. 3-2**, MS peaks corresponding to mono-sulfated glyceollin III/I ([Sul-glyceollin III/I – H]$^-$, 417.0650 *m/z*) and mono-glucuronized glyceollin III/ I ([GlcA-glyceollin III/I – H]$^-$, 513.1402 *m/z*) were detected in all the target organs as well as plasma at 0.5 h after administration. These conjugates were detected in the liver and the kidneys up to 6 h (**Fig. 3-2**), suggesting that administered glyceollins may possess a long-term retention ability at the organs in sulfated and/or glucuronized forms. As can be seen in EIC-MS chromatograms of each conjugate, observed broaden and doubled EICs of *e.g.,* mono-sulfated glyceollin I ([Sul-glyceollin I – H]$^-$, 417.0650 *m/z*) (**Fig. 3-2**) suggested that sulfated glyceollin I with different sulfated positions may occur at the organs. The accumulation behavior of conjugated glyceollins in organs was similar to production behavior of glyceollin metabolites in blood (**Fig. 2-8**).

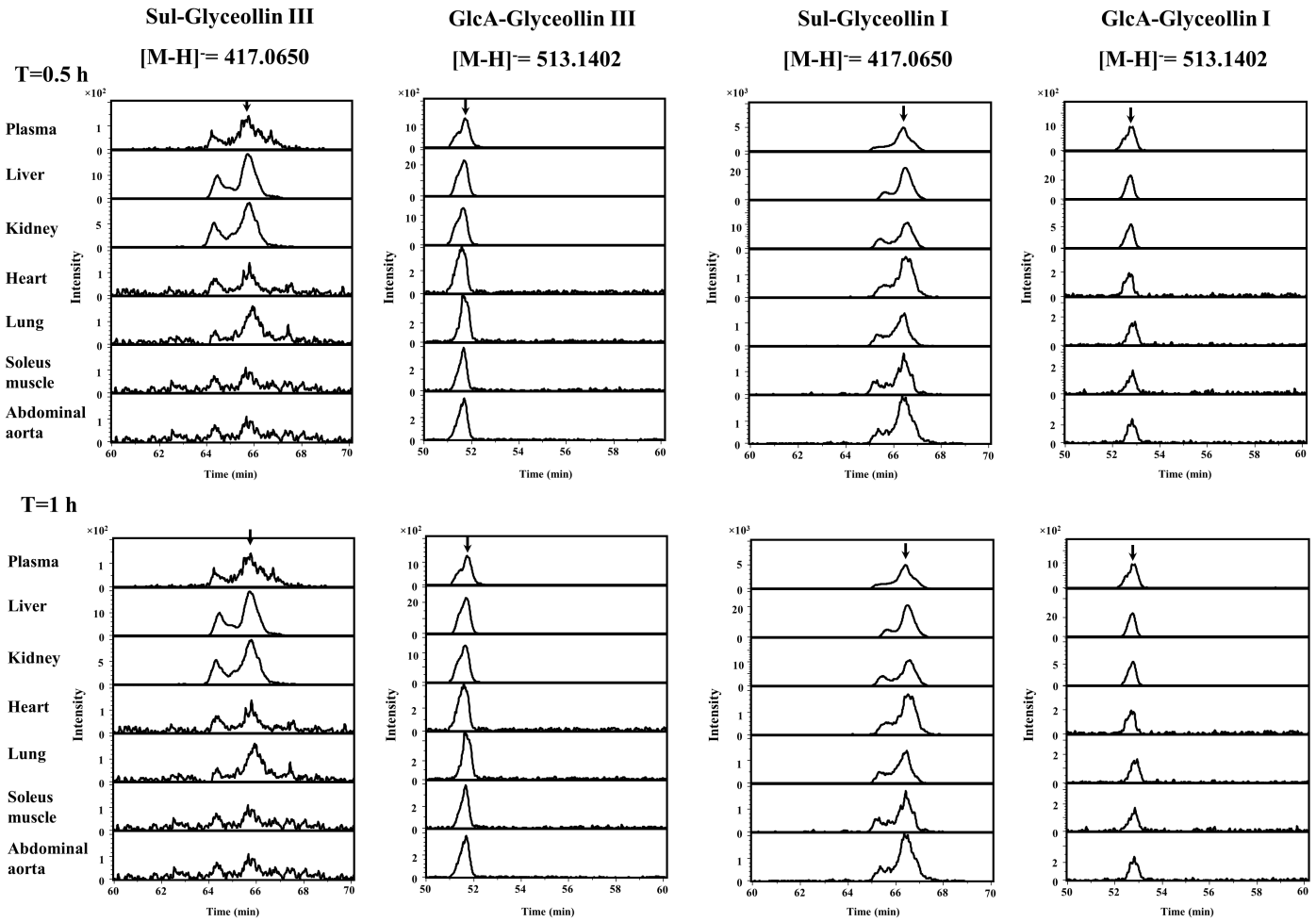


Fig. 3-2 LC-TOF/MS analysis of glyceollin conjugates in organs.

Organs of SD rat at 0.5, 1, 3, and 6 h after the administration (1.0 mg/kg) were subjected to LC-EIC-TOF/MS analysis. Sul-glyceollin III/I: $[M - H]^-$, 417.0650 *m/z,* GlcA-glyceollin III/I: $[M - H]^-$, 513.1402 *m/z.*

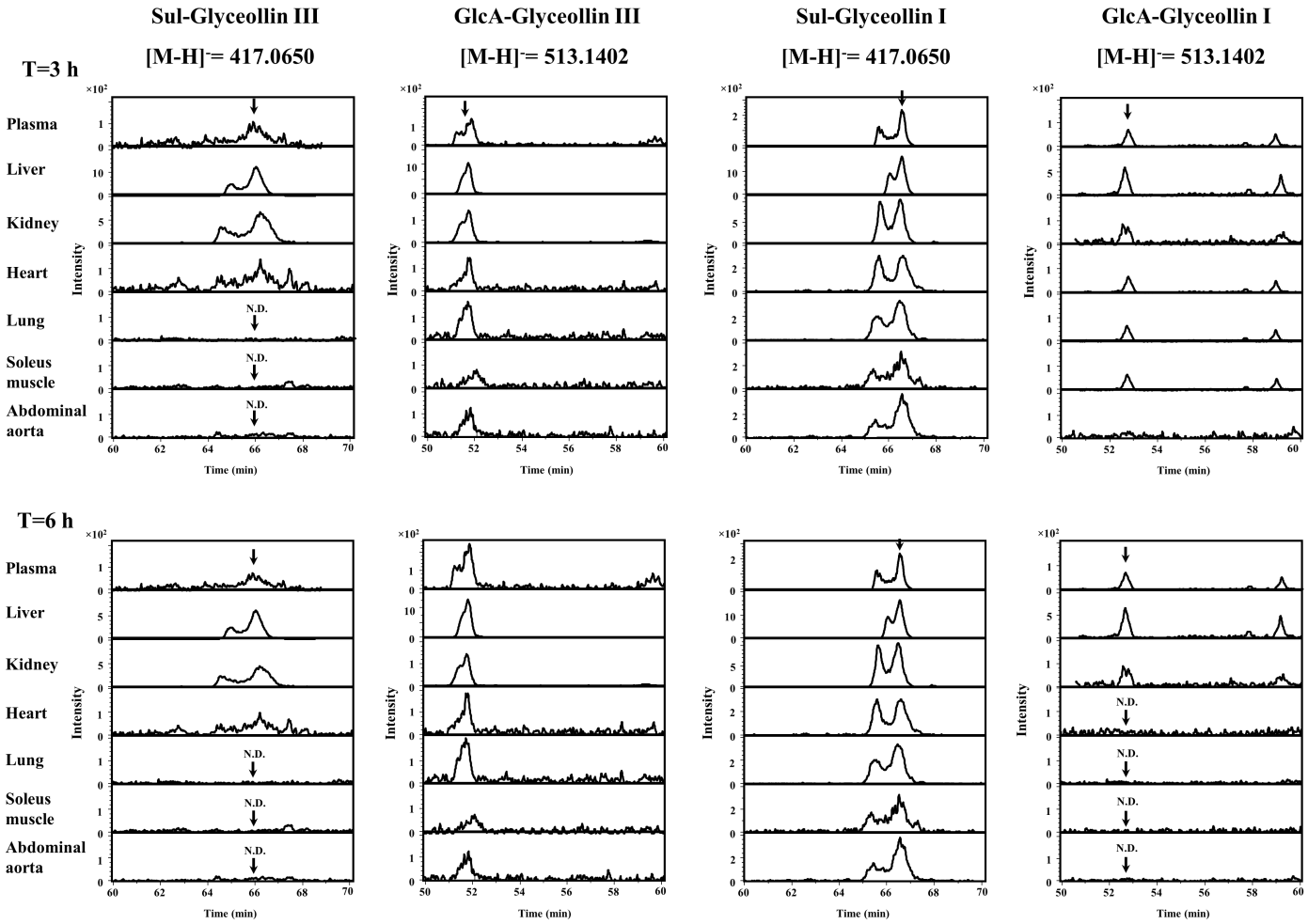


Fig. 3-2 (Continued)

The tissue distribution ratio of intact glyceollin III/I was estimated by the concentrations per total weight of organ in the whole rat body (**Fig. 3-3**). Since no standards were available for quantification of conjugates, sulfated glyceollin III/I, and glucuronized glyceollin III/I were tentatively estimated by MS peak area in total weight of organ by converting the peak area obtained in **Fig. 3-2.** As shown in **Fig. 3-3**, it was apparent that conjugates (sulfated and/or glucuronized forms) of the glyceollins III and I were significantly detected up to 6 h, indicating that glyceollins are preferably distributed and accumulated into the organs in conjugated forms, as similar to the rapid absorption in conjugated forms in the bloodstream (**Chapter II**). The ratio of the sulfated glyceollin I in the liver was higher than that of glucuronized glyceollin I, indicating that the metabolic speed of glyceollins to sulfated forms would be faster to glucuronidated forms. Considering the physiological potential(s) of sulfated metabolites in local tissues, *e.g.,* 3-*O*-sulfated vanillic acid [59] and 4-*O*-sulfated ferulic acid [60], in upregulating glucose transporter (GLUT) 4 and lowering elevated blood pressure in mice, respectively, sulfated glyceollins may also exert exert alternative physiological effects to those of intact glyceollins.

In this study, it was demonstrated that both types of conjugated (sulfated and/or glucuronized) forms of glyceollins were absorbed and accumulated into the circulatory organs (**Fig. 3-3**), while the tissue distribution is governed by complex physiological and biochemical processes, some reports demonstrated that the flavonoids might interact with organic anion transporters, such as monocarboxylate transporter (MCT)1 [98], organic anion transporter (OAT)1 [99], and organic anion transporting polypeptides (OATP)1B1 [100].

Considering that the structural and metabolically similarity of prenylated isoflavones, glyceollins, they may also be substrates and potent inhibitors of many members of the organic anion transporter family, while the tissue distribution route(s) of glyceollins and/or their conjugates remains unclear and further study is needed to clarify.

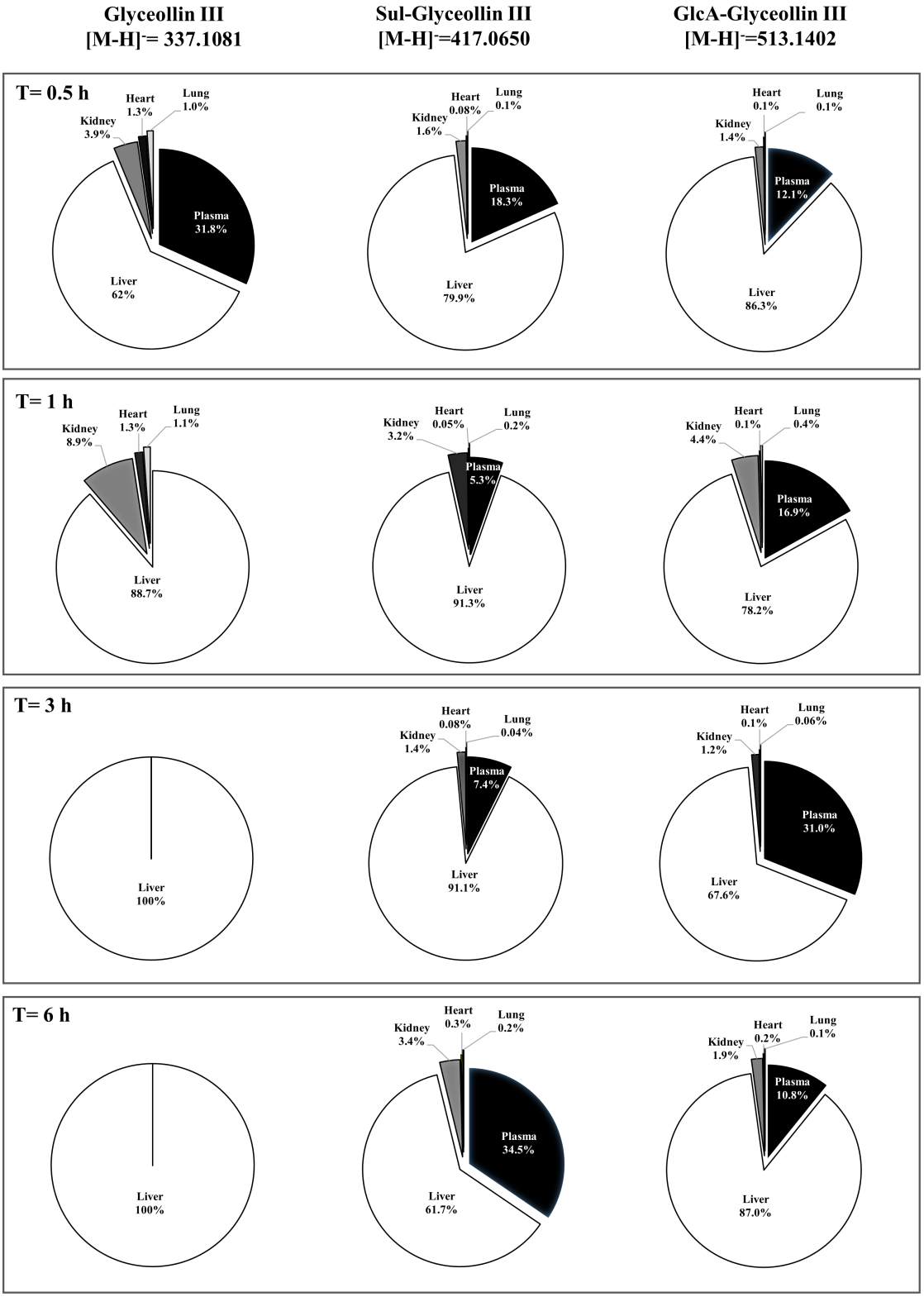


Fig. 3-3 Distribution patterns (percentage, %) of intact glyceollin III/I, Sul-glyceollin III/I,

and GlcA-glyceollin III/I in organs at 0.5, 1, 3, and 6 h after the administration to SD rats (1.0 mg/kg)

100% is the sum of the amount of glyceollins in plasma, the liver, the kidneys, the heart and the lungs at time points of T=0.5, 1, 3, 6 h. Each graph shows the percentage of glyceollins in plasma and organs (liver, kidney, heart, lung) relation to the total amount of glyceollins in them.

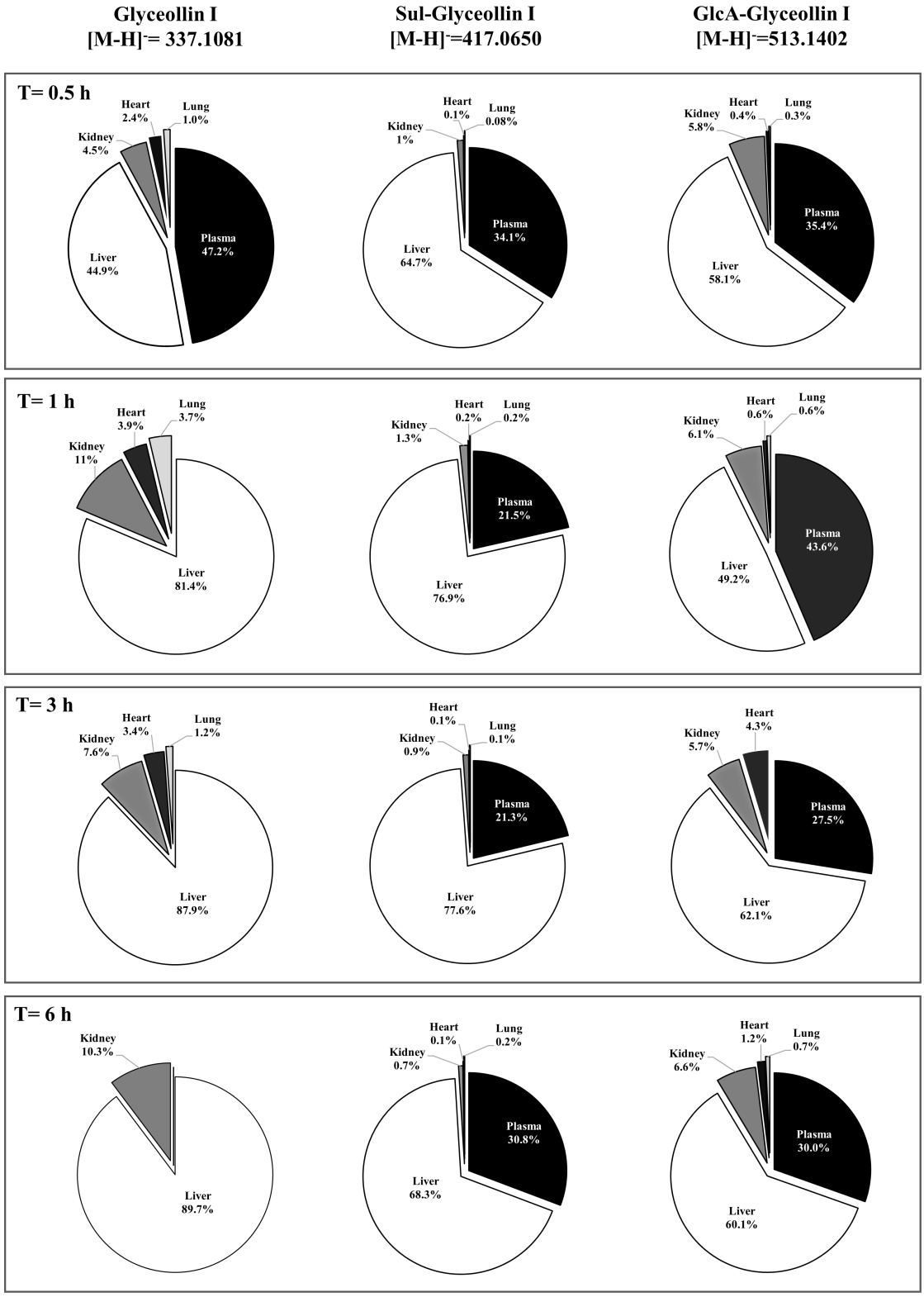


Fig. 3-3 (Continued)

4. Summary

In summary, **Chapter III** demonstrated the following findings: (1) upon oral administration to SD rats (1.0 mg/kg), glyceollins III and I were rapidly accumulated (T_{max} of 0.5 h) in the order of the liver > the kidneys > the heart > the lungs > the soleus muscle, the abdominal aorta; (2) glyceollins were more preferentially distributed than parent daidzein in circulatory organs as intact, sulfated or glucuronized forms up to 6 h after intake; (3) hydrophobic glyceollin I accumulation in organs was much higher than that of glyceollin III. In contrast, daidzein and equol-OH were detected only in the liver and the kidneys at less than 1/100-times lower amounts than those of glyceollins. The characteristic accumulation behavior of glyceollins in the organs (**Fig. 3-4**) provides scientific speculation on local physiological roles, as reported for other bioactive polyphenols, like EGCG showing protective effect in the liver [101], resveratrol showing cardioprotective effect in the heart [102], and daidzein eliciting myoblast differentiation and myotube growth in the muscle [103].

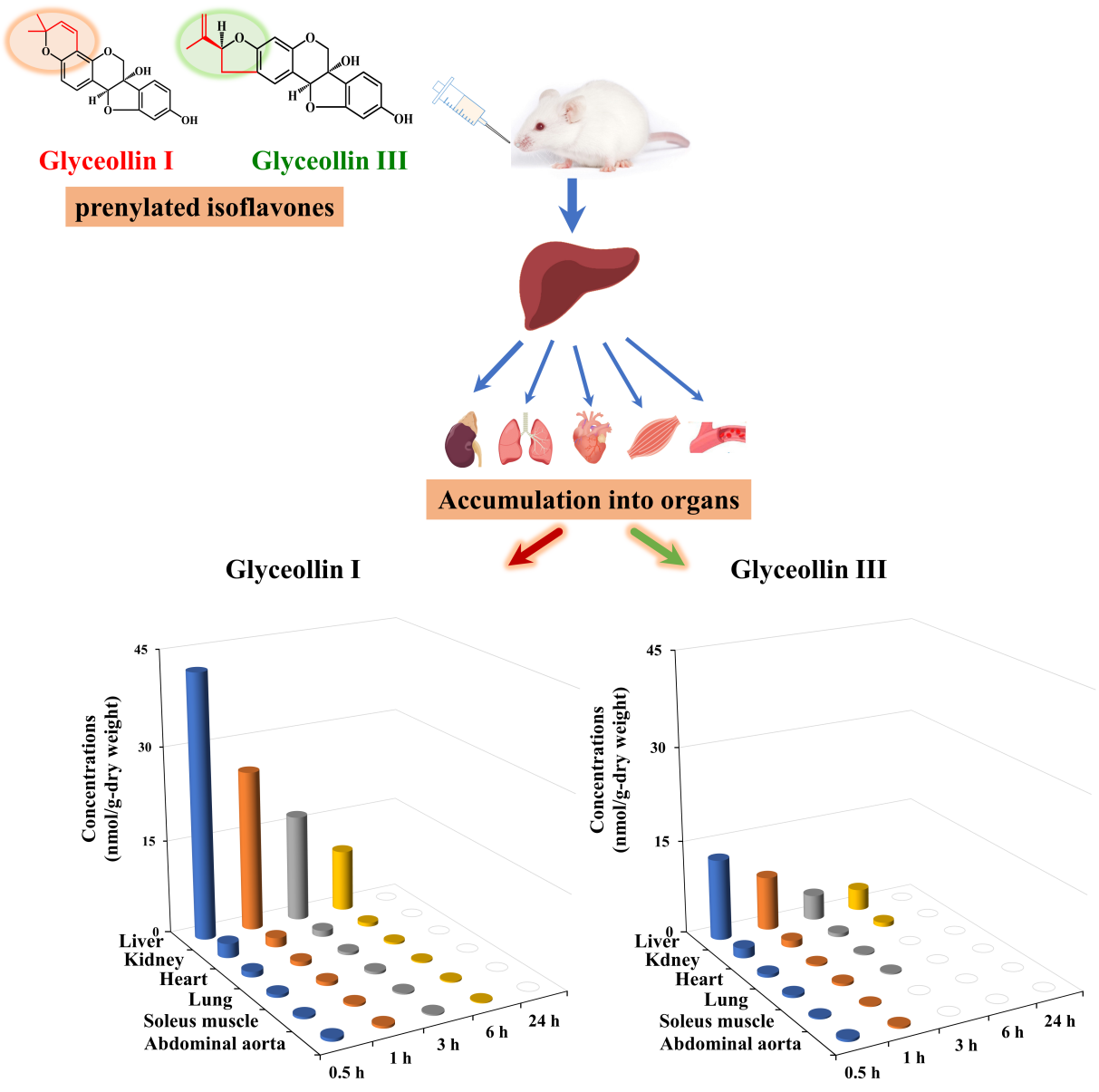


Fig. 3-4 Accumulation profiles of glyceollins in the organs of SD rat.

Chapter IV

Conclusion

Glyceollins are phytoalexins produced in soybeans in response to environmental (*e.g.* infection, wounding, freezing, ultraviolet light) or microbial stress [1-5]. Diverse *in vivo* physiological functions of glyceollins, *e.g.,* anti-oxidation [10], anti-tumor [11], anti-inflammation [12], and ER antagonistic effect [14-17] have been clarified so far. To understand the physiological actions of glyceollins, it is of importance to make clear the ADME in the body system. Thus, the present study aimed to get insights of bioavailability of prenylated isoflavones, glyceollins, in Sprague-Dawley (SD) rats in terms of intestinal absorption and subsequent tissue distribution.

Chapter II Intestinal absorption of prenylated isoflavones, glyceollins, in Sprague-Dawley rats

Although it was evidenced that prenylated isoflavones or glyceollins apparently elicit local physiological effects rather than isoflavones (*e.g.,* daidzein and genestein), the bioavailability remains unclear. In **Chapter II**, prenylated isoflavones (glyceollin III and I) and the parent isoflavone, daidzein, were targeted to clarify the intestinal absorption and metabolism in SD rats. Upon oral administration of 1.0 mg/kg glyceollin III or I to SD rats, no peaks corresponding to their intact forms were detected in the plasma by LC-TOF/MS analysis. In contrast, deconjugation treatment of plasma by sulfatase/β-glucuronidase enzymes resulted in a significant MS detection of glyceollins; absorption of conjugated forms of glyceollin I was much > 8-times higher than that of daidzein ($AUC_{0\text{-}8\,h}$: glyceollin I, 8.5 ± 0.7 nmol·h/mL; glyceollin III, 1.0 ± 0.2 nmol·h/mL; daidzein, 0.6 ± 0.1 nmol·h/mL), depending on their log *P* value or hydrophobicity. By MALDI-MS and LC-TOF/MS analyses of rat intestinal extract, major conjugates of glyceollins were methylation, sulfation, and glucronidation forms, while daidzein was mainly metabolized to form hydroxylated equol during intestinal absorption process. This demonstrated that prenylated isoflavones (glyceollins) are absorbed into the circulatory bloodstream in as conjugated forms, but not intact forms.

Taken together, it was demonstrated for the first time that the prenylation of isoflavones may promote intestinal absorption into rat bloodstream compared to their parent isoflavones, by increasing hydrophobicity. In addition, prenylated isoflavones, glyceollins, can be absorbed in conjugated forms such as methylation, sulfation, and glucuronidation, but not in intact forms.

Chapter III Tissue accumulation of prenylated isoflavones, glyceollins, in Sprague-Dawley rats

Apart from novel physiological effects of glyceollins, little is known about their bioavailability of tissue accumulation in organs. In **Chapter III**, the accumulation of orally administered prenylated isoflavones (glyceollin III and I) and the parent isoflavone, daidzein, into typical circulatory organs (the liver, the kidneys, the heart, the lungs, the soleus muscles, and the abdominal aorta) of SD rats were investigated by LC-TOF/MS. Upon oral administration of 1.0 mg/kg glyceollin III or I to SD rats, glyceollin I and III were accumulated up to 6 h in circulatory organs with T_{max} of 0.5 h, in the order of the liver > the kidneys > the heart > the lungs > the soleus muscles, the abdominal aorta. Hydrophobic glyceollin I accumulation in organs was higher than that of glyceollin III; daidzein and equol-OH were detected only in the liver and the kidneys at > 1/100-times lower amounts than those of glyceollins ($AUC_{0\text{-}6\ h}$ in the liver: glyceollin I, 113.2 ± 17.6 nmol·h/g-dry tissue glyceollin III, 32.5 ± 2.9 nmol·h/g-dry tissue; daidzein, 0.34 ± 0.05 nmol·h/g-dry tissue). Major conjugates accumulated in circulatory organs were sulfated and/or glucuronized forms up to 6 h after intake for glyceollins.

Taken together, **Chapter III** demonstrated firstly that prenylated isoflavones, glyceollins, were rapidly and preferably accumulated in circulatory organs within 0.5 h and remained stable and accumulated as sulfated and/or glucuronized forms up to 6 h after intake.

In conclusion, the present study demonstrated firstly the behavior in absorption of prenylated isoflavones, glyceollins, to rat circulating bloodstream. Absorbed glyceollins were received metabolic conjugation including methylation, sulfation, and glucuronidation during intestinal absorption process. In addition, the accumulation of orally administered glyceollins into typical circulatory organs (the liver, the kidneys, the heart, the lungs, the soleus muscles, and the abdominal aorta) of SD rats were investigated. The prenylated isoflavones, glyceollins, were preferentially distributed in the circulatory organs as intact, sulfated or glucuronized forms up to 6 h after the intake. Thus, the present study provides a novel scientific aspect on the high bioavailability of glyceollins by oral intake in preference to the intake of isoflavones.

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