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RESEARCH ARTICLE

Nutrikinetic modeling reveals order of genistein phase II metabolites appearance in human plasma

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Scope: Genistein from foods or supplements is metabolized by the gut microbiota and the human body, thereby releasing many different metabolites into systemic circulation. The order of their appearance in plasma and the possible influence of food format are still unknown. This study compared the nutrikinetic profiles of genistein metabolites.

Methods and results: In a randomized cross-over trial, 12 healthy young volunteers were administered a single dose of 30 mg genistein provided as a genistein tablet, a genistein tablet in low fat milk, and soy milk containing genistein glycosides. A high mass resolution LC-LTQ-Orbitrap FTMS platform detected and quantified in human plasma: free genistein, seven of its phase-II metabolites and 15 gut-derived metabolites. Interestingly, a novel metabolite, genistein-4'-glucuronide-7-sulfate (G-4'G-7S) was identified. Nutrikinetic analysis using population-based modeling revealed the order of appearance of five genistein phase II metabolites in plasma: (1) genistein-4',7-diglucuronide, (2) genistein-7-sulfate, (3) genistein-4'-sulfate-7-glucuronide, (4) genistein-4'-glucuronide, and (5) genistein-7-glucuronide, independent of the food matrix.

Conclusion: The conjugated genistein metabolites appear in a distinct order in human plasma. The specific early appearance of G-4',7-diG suggests a multistep formation process for the mono and hetero genistein conjugates, involving one or two deglucuronidation steps.

Keywords:

Genistein / Nutrikinetic modeling / Phase II metabolites



Additional supporting information may be found in the online version of this article at the publisher's web-site

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Abbreviations: AUC, area under the curve; FA, formic acid; FDR, false discovery rate; G-4'G-7S, genistein-4'-glucuronide-7-sulfate; G-4',7-diG, genistein-4',7-diglucuronide; G-4'G, genistein-4'-glucuronide; G-4'S, genistein 4'-sulfate; G-4'S-7G, genistein-4'-sulfate-7-glucuronide; G-7G, genistein-7-glucuronide; G-7S, genistein-7-sulfate; I_{\max} , maximum intensity; $t_{1/2}$, elimination half-life; t_{\max} , time between start and maximum intensity/concentration; t_{peak} , time between first appearance and maximum intensity; τ , lag time between start and first appearance; QC, quality control

1 Introduction

Soy isoflavones have been proposed to have protective effects in a variety of health conditions, including postmenopausal osteoporosis [1], alleviation of menopause-related hot flashes [2], risk reduction of certain cancers [3], and cardiovascular

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events [4]. A number of these benefits are attributed to the phytoestrogen activity of isoflavones such as genistein and daidzein. In view of these health benefits isoflavones are attractive to be used in dietary supplements. In unprocessed soybeans or in soy foods, isoflavones are mainly present as conjugates in the form of glucosides, but also as acetyl- or malonyl-glycosides.

Only the unconjugated forms of isoflavones are absorbed by the human body [5]. Interestingly, in unprocessed soybeans and some soy products the genistein aglycone is only found in low concentrations, while its glucoside conjugate, genistin, is much more abundant [6]. Nevertheless, in a number of soy foods the genistein aglycone is present in elevated amounts. The glucoside forms of isoflavones in food are hydrolyzed in the intestine before crossing the gut mucosa. Similar to daidzein that is metabolized by intestinal conversion to equol, it has recently been proposed that genistein is transformed by the gut microflora to 5-hydroxy-equol [7]. However, only a percentage (25–60%) of soy consumers is able to form equol out of daidzein. The presence of specialized intestinal bacteria is needed for the conversion of daidzein into dihydrodaidzein to form equol [8]. Analogously, it has been postulated that genistein is metabolized via dihydrogenistein to form 5-hydroxy-equol, when 5-hydroxy-equol producing bacteria copopulate the gut, but so far this has only been demonstrated in mice, not in humans [7,9]. In the nineties, different groups have explored the catabolic pathway of genistein [10–13]. They proposed a metabolic genistein biotransformation by the gut microbiota that goes through the catabolic metabolites dihydrogenistein, 6'-hydroxy-*O*-desmethylangolensin, 4-hydroxyphenyl-2-propionic acid, and 4-ethylphenol.

Besides the gut-derived genistein catabolites, unconjugated genistein is absorbed in the intestine and to a smaller extent also in the colon [14–16]. A large part of the absorbed genistein enters the portal vein in the form of glucuronides and sulfates formed by phase II enzymes in the intestine and colon. The remaining genistein that enters the portal vein is metabolized by UDP-glucuronosyltransferases and sulfotransferases in the liver. The liver releases the genistein glucuronides and sulfates and they either go through the enterohepatic cycle or into the sinusoidal blood stream [17–20]. In man only a few percent of unconjugated genistein will enter the circulation [21], whereas in rodents this fraction can be much higher [22]. Interestingly, it has been shown that genistein glucuronides can be hydrolyzed back into genistein aglycone by cellular enzymes, whereas sulfates cannot [16].

Some human studies comparing the absorption of pure genistein with that of conjugated glycosides showed greater bioavailability when ingested as beta-glycosides rather than as aglycone, as measured from the area under the curve (AUC) [23,24], while one study showed identical bioavailability [25]. In all studies the elimination half-lives ($t_{1/2}$) and the mean residence time of the total genistein were similar between both aglycone and glycoside forms. Although differences in absorption between the two forms were noted by Yuan et al.,

the C_{\max} and AUC_{0-24h} values after glycoside or aglycone intake were not significantly different for all metabolites.

The phase II metabolites of genistein in human plasma are glucuronidated and sulfated forms at positions 4' and/or 7. The major conjugated metabolites identified in human plasma were genistein-4'-sulfate-7-glucuronide (G-4'S-7G), genistein-4',7-diglucuronide (G-4',7-diG), genistein-7-sulfate (G-7S), genistein-4'-glucuronide (G-4'G), genistein-7-glucuronide (G-7G), genistein-4'-sulfate (G-4'S), and genistein-4',7-disulfate (G-4',7-diS), whereas G-4'S and G-4',7-diS were extremely low abundant [24,26,27]. Together with gut-derived metabolites dihydrogenistein, 6'-hydroxy-*O*-desmethylangolensin, 4-hydroxyphenyl-2-propionic acid, and 4-ethylphenol they represent the metabolic fate of genistein in the ecosystem of the human superorganism [28].

In this study, we aimed to explore the nutrikINETIC pattern of the metabolic fate of genistein conjugates and metabolites as detected by high mass resolution HPLC-Orbitrap FTMS in plasma from 12 healthy individuals, after oral consumption of genistein tablets, genistein in low-fat milk or soy milk. For this study, we employed the nutrikINETICS concept [28,29] and investigated the order of appearance of the different genistein derivatives by using population-based modeling.

2 Materials and methods

2.1 Subjects, study design, and test formats

This study was a prospective, 3-periods cross-over, randomized, single-dose trial conducted at one study center (Galmed, Halle, Germany). The study was conducted in an open, unblinded fashion, the treatment periods were separated by a washout of at least 7 days. The study was conducted in 12 healthy Caucasian subjects (six male, six female, 18–40 years, BMI 18–28, nonsmokers/smokers up to ten cigarettes per day). Subjects that were participants in any other study or donated blood during the last 30 days before start, with known hypersensitivity or allergy to soy, peanuts, purified isoflavones, genistein, lactose, and/or cow milk or had preexisting chronic diseases or were taking drugs were excluded. Soy-based foods or isoflavone supplements were prohibited prior and during the study. The study was approved by the independent ethics committee of the General Medical Council of Land Saxony-Anhalt and was conducted according to the principles of the international conference on harmonization of technical requirements for registration of pharmaceuticals for human use (ICH) good clinical practice (GCP) according the declaration of Helsinki (ICH-GCP). A signed informed consent was obtained from the participants.

Pure genistein (min. 98.5% genistein, supplied by DSM Nutritional Products, Switzerland) was granulated and compressed into tablets and packaged by SwissCo, Switzerland

under GMP requirements and control. Samples were retained and stored at the sponsor's site. According to a randomization scheme the subjects (each treatment period, three groups of $n = 4$) were administered a genistein tablet with 200 mL of tap water or a tablet with ~ 500 mL low-fat cow milk (referred to as "low-fat milk") or 500 mL soy milk. All three formats contained 30 mg genistein, only in the soy milk genistein was present as a glycoside (genistin). The subjects were required to drink it within 5–10 min. The subjects were housed in a clinical study test facility throughout the pharmacokinetic study in controlled setting that included standardized meals at defined times for all subjects. Ten milliliters of venous blood was drawn into an EDTA plasma-tube at each sampling point. It was centrifuged with 1550 g (3000 rpm) for 10 min at 4°C to collect plasma. Blood sampling was at the time points –12 and –0.5 h before and 0.5, 1, 1.5, 2, 3, 4, 5, 6, 8, 9, 10, 13, 16, 24, 36, 48 h after administration of the single-dose treatment. Seven time points, –0.5, 3, 5, 9, 16, 24, and 36 h, were included in the nutrikinetic study described in this paper.

Safety and tolerability of the genistein tablets administered as a single oral dose of 1×30 mg genistein with 12 healthy subjects was good. No clinically relevant abnormalities were observed in the medical examinations and in the laboratory investigations. The study was completed without any dropout. Two adverse events were reported (headache and abdominal pain in relation to menstruation) after administration of soy milk. Any relation to the soy milk was not given. After intake of the genistein tablets no adverse event was observed.

2.2 Reagents and chemicals

Methanol and acetonitrile (HPLC grade) were purchased from BioSolve BV (Valkenswaard, the Netherlands), formic acid (p.a. grade) from Merck (Darmstadt, Germany). Genistein was purchased from Extrasynthese (Genay, France). Genistein 4'- β -D-glucuronide, genistein 7- β -D-glucuronide, and genistein 7- β -D-glucuronide 4'-sulfate from Toronto Research Chemicals (Ontario, Canada). Daidzein, phloroglucinol (1,3,5 trihydroxybenzene), pyrogallol, 2(4-hydroxy phenyl)-propionic acid, and gallic acid were purchased from Sigma-Aldrich (St Louis, USA). The commercial human plasma (from single donors aged between 18 and 65) used as quality control (QC) was purchased from Innovative Research (Michigan, USA – Cat#: IPLA-N).

2.3 Sample preparation and LCMS analysis

Plasma extracts were prepared as follows: 1.2 mL of extraction mix composed of 83.3% ACN, 1.3% v/v of formic acid (FA) and 2.9 mM of vitamin C was added to 0.4 mL of plasma sample. The sample was immediately vortexed and allowed to rest for 20 min on ice and then sonicated for 15 min and centrifuged for 10 min at 2500 rpm. The supernatant was firstly

partially dried in a speed-vac for 1 h, to remove excess of ACN, and then completely dried overnight in a freeze dryer. The residue was reconstituted in 100 μ L of 50% methanol in 0.1% FA, briefly vortexed, allowed to stand on ice for 20 min, sonicated for 15 min and centrifuged again for 20 min. The supernatant was filtered through a 0.45 μ m inorganic membrane filter and finally transferred into an HPLC vial with glass inset.

The LC-MS system consisted of an Accela HPLC system with an Accela photodiode array (PDA) detector connected to a LTQ/Orbitrap FTMS hybrid mass spectrometer (Thermo Fisher Scientific) equipped with an ESI source and controlled by Xcalibur 2.1 software. The injection volume was 5 μ L and LC separation was achieved on a Luna C18(2) analytical column (150 \times 2 mm, 3 μ m particle size, Phenomenex, Torrance, CA). The mobile phase A consisted of MQ water and the mobile phase B of ACN, both acidified with 0.1% FA. The flow rate was 0.19 mL/min, column and sample organizer were set up at 40°C and 4°C, respectively. The linear gradient started at 5% and increased to 35% B in 45 min. Then, 15 minutes of washing and equilibration before next sample injection was programmed, resulting in a total run time of 60 min.

The MS analysis was carried out in ESI-negative mode. Data were collected at m/z 100–1000 with a resolution of 60,000 (at m/z 400) in centroid mode. The capillary temperature was 295°C, sheath gas flow was set at 50 arbitrary units (a.u.), auxiliary gas flow at 5 a.u. and the sweep gas flow at 5 a.u. The Orbitrap was externally calibrated in negative mode using sodium formate clusters in the range m/z 150–1200 and automatic tuning was performed on m/z 384.93. AGC target value of the Ion Trap was set at 30 000 charges. The ion tube was cleaned between each sample batch, in order to retain sensitivity.

Samples were divided into 12 batches of 21 study samples, each batch corresponding to all seven selected time points of the three treatments per volunteer. Each injection batch corresponded to these 21 study samples injected in a random order plus three commercial plasma QCs samples analyzed in the beginning, middle, and end of the sample series.

2.4 Data preprocessing

The identification of genistein, genistein conjugates, daidzein, and phenolic compounds in the study samples was based on corresponding accurate mass (within 2 ppm accuracy) and retention times compared with the reference compounds. For this purpose, the raw data files were all preprocessed (peak picking by unbiased baseline correction and noise estimation) using metAlign software [30]. MetAlign output files (.redms_acc format) were used for ultrafast mass_retention time searching using the Search_LCMS module within the MetAlign software. Output files were in .csv format and contained accurate mass, retention time, scan

Table 1. Metabolites included in the nutrikinetic analyses

No.	Metabolite	Group of metabolites
1	Free genistein	Intact isoflavone
2	Genistein 4'-glucuronide (G-4'G)	Phase II metabolite
3	Genistein 7-glucuronide (G-7G)	Phase II metabolite
4	Genistein 4'-sulfate (G-4'S)	Phase II metabolite
5	Genistein 7-sulfate (G-7S)	Phase II metabolite
6	Genistein 4'-sulfate-7-glucuronide (G-4'S-7G)	Phase II metabolite
7	Genistein 4'-glucuronide-7-sulfate (G-4'G-7S)	Phase II metabolite
8	Genistein diglucuronide (G-4',7-diG)	Phase II metabolite
9	Dihydrogenistein	Gut-mediated metabolite
10	Benzoic acid	Gut-mediated metabolite
11	Phloroglucinol (1,3,5-trihydroxybenzene)	Gut-mediated metabolite
12	Pyrogallol	Gut-mediated metabolite
13	3-OH Benzoic acid	Gut-mediated metabolite
14	2,4-Dihydroxy benzoic acid	Gut-mediated metabolite
15	2(4-Hydroxyphenyl)propionic acid	Gut-mediated metabolite
16	Gallic acid	Gut-mediated metabolite
17	Hippuric acid	Gut-mediated metabolite
18	2,6-Dimethoxy benzoic acid	Gut-mediated metabolite
19	p-Cresol-sulfate	Gut-mediated metabolite
20	Resorcinol sulfate	Gut-mediated metabolite
21	4-Ethylphenol sulfate	Gut-mediated metabolite
22	5-Hydroxy-equol	Gut-mediated metabolite
23	5-(3,4-Dihydroxyphenyl)- γ -valerolactone	Gut-mediated metabolite

number, ppm mass error, and signal intensity (peak height) for each target compound in each sample.

Analytical variation like decreasing system sensitivity, column degradation, MS source pollution, sample degradation etc. during the analysis of a batch of samples was monitored by the inclusion of QC samples, i.e. samples that have a fixed composition and analyzed at specified intervals within that batch of samples [31]. These QC samples were placed at the beginning, middle, and end of each analytical batch. It was assumed that the same analytical variation was present in the measurements of the QC and study samples.

2.5 Noncompartmental plasma kinetic analysis

Noncompartmental plasma kinetic analysis for each genistein metabolite (Table 1) included evaluation of the maximum intensity levels (I_{\max}), time to reach I_{\max} (t_{\max}), and the area under the curve of metabolite intensity versus time

($AUC_{[0-t]}$). I_{\max} and t_{\max} were read directly from the observed curves. $AUC_{[0-t]}$ was calculated according to the linear trapezoidal rule from 0 hour (intensity level measured at $t = -0.5$ h) to the last quantifiable intensity level after treatment administration (intensity level measured at $t = 36$ h). Terminal elimination half-time ($t_{1/2}$) was calculated as $(\ln 2)/k_{el} = 0.693/k_{el}$, where k_{el} is the terminal rate constant calculated by least-squares regression of the terminal semi-logarithmic intensities versus time data.

Descriptive statistics were calculated for I_{\max} , t_{\max} , $AUC_{[0-t]}$, and $t_{1/2}$ on the basis of unchanged (t_{\max} , $t_{1/2}$) and log transformed data (I_{\max} and $AUC_{[0-t]}$). They included mean \pm SD. Wilcoxon signed rank test for zero median ($p < 0.05$) was used to assess differences between three different treatments. False discovery rate (FDR) correction of obtained p -values by Benjamini–Hochberg was applied with $q = 0.05$.

2.6 Selection of metabolites for population-based nutrikinetic modeling

Metabolite intensity versus time-curves of all metabolites included in the study were visually inspected (see Supporting Information Fig. 1). Metabolites detected in at least three time points in at least eight out of the 12 subjects and present in all treatment groups, and with reasonable shapes of their intensity versus time-curves were selected for further statistical analysis. This selection process yielded five metabolites: G-4'G, G-7G, G-7S, G-4'S-7G, and G-4',7-diG. Data of volunteers 1, 2, and 4 were excluded from further nutrikinetic modeling, because the curves were very much deviating from other subjects and from first-order absorption and elimination.

2.7 Population-based nutrikinetic modeling

A one-compartment kinetic model with first-order absorption and elimination (equation (1)) was used to describe the time courses $\gamma(t)$ of the genistein phase II metabolites in plasma (metabolites selected as described in a previous section) at a given dose D [32]. This kinetic model had four parameters: τ , lagtime; V , volume of distribution; k_a , absorption rate constant; k_{el} , elimination rate constant.

$$E(\gamma(t)) = \frac{D}{V} \frac{k_a}{k_a - k_{el}} (e^{-k_{el}(t-\tau)} - e^{-k_a(t-\tau)}), \quad t \geq \tau$$

$$E(\gamma(t)) = 0, \quad t < \tau$$
(1)

The parameters were estimated in a population modeling approach using nonlinear-mixed effects models using Monolix 3.1 software applied in Matlab R2010b (MONOLIX, 3.1 User Guide, software.monolix.org; [33]). An individual

parameter vector $p_{ik} = [\tau_{ik} \ k_{a_{ik}} \ V_{ik} \ k_{el_{ik}}]$ (subject index $i = 1 \dots N$; treatment index $k = 1, \dots, K$) is expressed as

$$\log(p_{ik}) = \mu + \beta_k + b_i + c_{ik} \quad (2)$$

where $\mu + \beta_k$ represents the fixed effect for treatment k , $b_i \sim N(0, \Omega)$ the random effect of subject i and $c_{ik} \sim (0, \Gamma)$ the random effect of subject i and treatment k . The random effects are assumed to be mutually independent and the total variance is thus decomposed into the between-subject variance, Ω , and the within-subject variance, Γ [34]. A constant additive error model is assumed for the residual error.

SDs of the population estimates, sd_{pk} , were calculated from leave one subject out jackknife estimates according to [35]:

$$sd_{pk} = \sqrt{\frac{N-1}{N} \sum_i (p_{k\setminus i} - \bar{p}_k)^2} \quad (3)$$

Where p_k represents the population parameters $[\tau_k \ k_{a_k} \ V_k \ k_{el_k}]$ for treatment k , $p_{k\setminus i}$ is the population estimate without subject i , and $\bar{p}_k = \frac{1}{N} \sum_i p_{k\setminus i}$.

3 Results

3.1 Genistein and gut-derived metabolites

The LC-LTQ-Orbitrap FTMS platform applied at high mass resolution in full-scan ESI-negative mode enabled the detection of both genistein aglycone and 22 isoflavonoid-related metabolites within a mass accuracy of 2 ppm and in most cases with specific mass fragments and/or coelution with authentic standards. See Table 1 for the list of compounds used in the further data analyses, and see also Supporting Information Table 1 for detailed LCMS information of all target compounds and their identification level according to the Metabolomics Society Initiative [36].

During the analysis, in most batches we observed a clear decrease in total intensity, which is the sum of all mass signals in the chromatogram as determined by the data preprocessing software of the three QC samples during the analysis time of a sample batch (see Supporting Information Fig. 2 shown by red crosses). This indicated a gradual loss of analytical sensitivity in time, due to contamination of the ion source by the concentrated plasma extract. A large difference between the average total intensity of the QC samples per batch was also observed, indicating batch-dependent variation in extraction and/or analytical sensitivity. To compensate for between and within batch variation, a QC-based batch-wise correction of peak intensities was successfully applied [37]. The batch correction factor applied was equal for all metabolites detected in that specific batch. The QC-corrected metabolites intensities were further used to explore their nutrkinetic patterns by noncompartmental analysis and population-based nutrkinetic modeling.

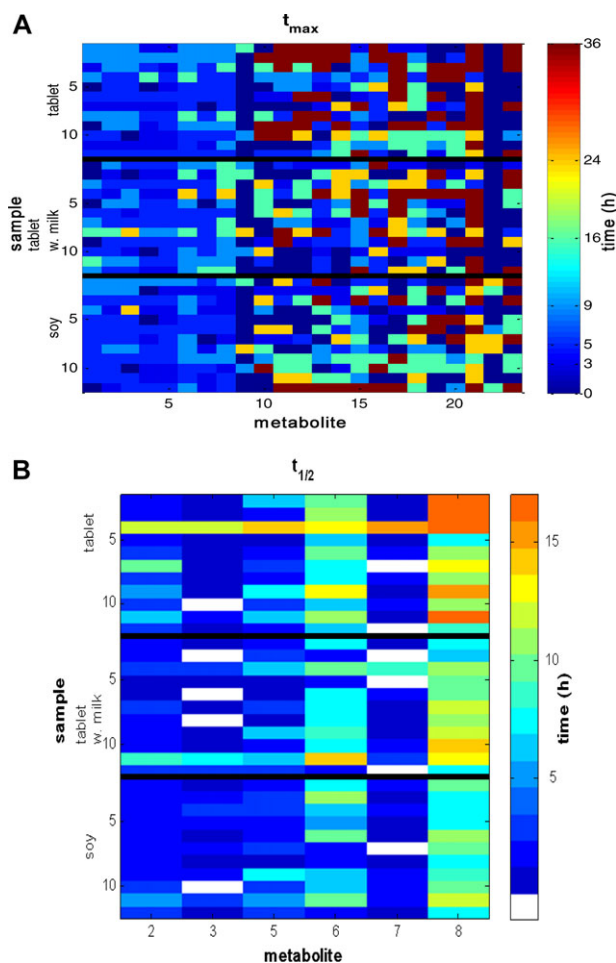


Figure 1. Overview of t_{max} (A) and $t_{1/2}$ (B) observed for 23 genistein metabolites in 12 (11 for $t_{1/2}$) subjects after three different treatments: genistein tablet, genistein tablet with cow milk, and soy milk. The numbering of the metabolites corresponds to the numbering in Table 1. The values are color coded and grouped by treatment. Similarly, Fig. 1B shows $t_{1/2}$ values for the subjects that were estimated only for six of the seven human phase II metabolites of genistein (metabolites 2, 3, 5, 6, 7, and 8) because only for these metabolites linear relationship between terminal semi-logarithmic intensities and time could be obtained. Subject no. 4 was excluded from the $t_{1/2}$ analysis for the same reason.

3.2 Noncompartmental analysis

Noncompartmental analysis was composed of the evaluation of the maximum intensity levels (I_{max}), time to reach I_{max} (t_{max}), the area under the curve of metabolite intensity versus time ($AUC_{[0-t]}$) and terminal elimination half-time ($t_{1/2}$). The overview of t_{max} and $t_{1/2}$ values for all metabolites and subjects included in the study is given in Fig. 1. Results of Wilcoxon signed rank test, performed to compare different treatments for I_{max} , t_{max} , AUC , and $t_{1/2}$, are presented in Table 2.

Analysis of I_{max} and $AUC_{[0-t]}$ has shown that some metabolites are present only for some subjects, e.g. dihydrogenistein

Table 2. Wilcoxon signed rank test p -values for between treatments comparison of nutrikINETIC parameters: I_{\max} , t_{\max} , AUC, and $t_{1/2}$ determined for genistein and its metabolites during noncompartmental analysis

Metabolite ^{a)}	I_{\max} ^{b)}			t_{\max}			AUC			$t_{1/2}$		
	A–B ^{c)}	A–C	B–C	A–B	A–C	B–C	A–B	A–C	B–C	A–B	A–C	B–C
1	>0.05	>0.05	0.0068	>0.05	>0.05	>0.05	>0.05	>0.05	>0.05	>0.05	>0.05	>0.05
2	>0.05	>0.05	0.0068	>0.05	>0.05	>0.05	>0.05	>0.05	0.0342	0.0049	0.0244	>0.05
3	>0.05	>0.05	0.0210	>0.05	>0.05	>0.05	>0.05	>0.05	>0.05	>0.05	>0.05	>0.05
4	>0.05	>0.05	>0.05	>0.05	>0.05	>0.05	>0.05	>0.05	0.0425	0.0186	0.0322	>0.05
5	>0.05	>0.05	>0.05	>0.05	>0.05	>0.05	>0.05	>0.05	>0.05	>0.05	>0.05	>0.05
6	>0.05	0.0425	0.0005	>0.05	>0.05	>0.05	>0.05	0.0122	>0.05	0.0010	>0.05	>0.05
7	>0.05	>0.05	>0.05	>0.05	>0.05	>0.05	>0.05	>0.05	>0.05	>0.05	>0.05	>0.05
8	0.0010	>0.05	0.0005	0.0156	0.0156	0.0020	0.0005	>0.05	0.0010	>0.05	>0.05	>0.05
9	>0.05	>0.05	>0.05	>0.05	>0.05	>0.05	>0.05	>0.05	>0.05	>0.05	>0.05	>0.05
10	>0.05	>0.05	>0.05	>0.05	>0.05	>0.05	>0.05	>0.05	>0.05	>0.05	>0.05	>0.05
11	>0.05	>0.05	>0.05	>0.05	>0.05	>0.05	>0.05	>0.05	>0.05	>0.05	>0.05	>0.05
12	>0.05	>0.05	>0.05	>0.05	0.012	>0.05	>0.05	>0.05	>0.05	>0.05	>0.05	>0.05
13	>0.05	>0.05	>0.05	>0.05	>0.05	>0.05	>0.05	>0.05	>0.05	>0.05	>0.05	>0.05
14	0.0024	>0.05	>0.05	>0.05	>0.05	>0.05	0.0010	>0.05	>0.05	>0.05	>0.05	>0.05
15	>0.05	>0.05	>0.05	>0.05	>0.05	>0.05	>0.05	>0.05	>0.05	>0.05	>0.05	>0.05
16	>0.05	>0.05	>0.05	>0.05	>0.05	>0.05	>0.05	>0.05	>0.05	>0.05	>0.05	>0.05
17	>0.05	>0.05	>0.05	>0.05	>0.05	>0.05	>0.05	>0.05	>0.05	>0.05	>0.05	>0.05
18	>0.05	>0.05	>0.05	>0.05	>0.05	>0.05	>0.05	>0.05	>0.05	>0.05	>0.05	>0.05
19	>0.05	>0.05	>0.05	>0.05	>0.05	>0.05	>0.05	>0.05	>0.05	>0.05	>0.05	>0.05
20	>0.05	>0.05	>0.05	>0.05	>0.05	>0.05	>0.05	>0.05	>0.05	>0.05	>0.05	>0.05
21	>0.05	>0.05	>0.05	>0.05	>0.05	>0.05	>0.05	>0.05	>0.05	>0.05	>0.05	>0.05
22	0.0313	>0.05	0.0313	0.0313	>0.05	0.0313	0.0313	>0.05	>0.05	>0.05	>0.05	>0.05
23	>0.05	>0.05	>0.05	>0.05	>0.05	>0.05	>0.05	>0.05	>0.05	>0.05	>0.05	>0.05

a) The numbering of the metabolites corresponds to the numbering given in Table 1.

b) Only p -values lower than 0.05 are given, p -values that indicate significant difference also after false discovery correction are indicated in bold.

c) Treatments: A: genistein tablet; B: soy milk; C: genistein tablet with low-fat cow milk.

is present only for subject 9 and some metabolites are only present after soy milk treatment: 3-hydroxy benzoic acid and 5-hydroxy equol. Statistically significantly higher I_{\max} in soy milk treatment group were found for genistein, G-4'G, G-7G, G-4'S-7G when compared with I_{\max} of tablet with low-fat milk group. This was also the case for G-4',7-diG when compared to both tablet and tablet with low-fat milk group and for 2,4-dihydroxy benzoic acid when compared to the tablet group. After FDR correction differences in genistein, G-4'G, G-4'S-7G, G-4',7-diG, and 2,4-dihydroxy benzoic acid were still statistically valid. Statistically significant higher $AUC_{[0-t]}$ in the soy milk treatment group were found for G-4'S-7G, G-4',7-diG, and 2,4-dihydroxy benzoic acid when compared with the tablet group and for G-4'G, G-4'S, G-4'S-7G, and G-4',7-diG when compared to the tablet with low-fat milk group. After FDR correction differences in $AUC_{[0-t]}$ of G-4'S-7G, G-4',7-diG, and 2,4-dihydroxy benzoic acid were still statistically valid. However, no difference was observed between genistein tablet and genistein tablet with low-fat milk. All in all, the most significant differences were seen for phase II metabolites. Unfortunately, for the gut-mediated metabolites the interindividual variability was too high to perform such noncompartmental data analysis.

The t_{\max} of genistein and most human phase II metabolites of genistein ranged between 3 and 9 h (Fig. 1A). In contrast, t_{\max} of gut-mediated metabolites of genistein varied from $t = 0$ h to $t = 36$ h. We observed a large interindividual variation in t_{\max} values, especially for the gut-mediated metabolites. The t_{\max} of G-4',7-diG was statistically significantly shorter in the soy milk treatment group when compared with the tablet-with-milk group.

The $t_{1/2}$ values for the subjects were estimated for six of the seven human phase II metabolites of genistein (metabolites 2–3 and 5–8) because only for these metabolites a linear relationship between terminal semi-logarithmic intensities and time could be obtained. For subject 4, there was no linear relationship between terminal semi-logarithmic intensities and time for none of the metabolites, and this subject was therefore excluded from the $t_{1/2}$ analysis. Six phase II metabolites of genistein differed in their $t_{1/2}$ values (Fig. 1B), ranging from 1 to 36 h. The $t_{1/2}$ of the single-conjugated genistein forms, either glucuronidated or sulfated, were shorter than its double-conjugated forms, like glucuronide-sulfates and diglucuronides. The $t_{1/2}$ of G-4'G, G-4'S-7G, and G-4',7-diG were significantly shorter in the soy milk treatment group when compared with tablet group, also after FDR correction.

Table 3. Population estimates for τ , t_{peak} , and the sum of these (t_{max}) for five phase II metabolites

Metabolite ^{a)}	τ (h)	SD τ (h) from jack- knife	t_{peak} (h)	SD t_{peak} (h) from jack- knife	t_{max} (h) ^{b)}
Genistein tablet					
Genistein-4',7 diglucuronide (G-4',7-diG)	0.91	0.73	7.93	1.51	8.84
Genistein 7-sulfate (G-7S)	1.94	0.79	1.34	0.54	3.31
Genistein 4'-sulfate-7-glucuronide (G-4'S-7G)	2.53	0.27	3.38	1.08	5.91
Genistein 4'-glucuronide (G-4'G)	2.80	0.50	2.02	0.84	4.82
Genistein 7-glucuronide (G-7G)	3.07	0.44	1.44	0.56	4.51
Soy milk					
Genistein 4',7diglucuronide (G-4',7-diG)	1.05	0.73	3.52	0.97	4.57
Genistein 7-sulfate (G-7S)	2.47	0.85	1.47	0.60	3.94
Genistein 4'-sulfate-7-glucuronide (G-4'S-7G)	2.64	0.32	3.40	1.01	6.04
Genistein 4'-glucuronide (G-4'G)	3.08	0.39	1.29	0.28	4.37
Genistein 7-glucuronide (G-7G)	3.58	0.34	0.66	0.25	4.23
Genistein tablet with low-fat cow milk					
Genistein 4',7 diglucuronide (G-4',7-diG)	0.65	0.69	9.53	1.11	10.19
Genistein 7-sulfate (G-7S)	1.91	0.99	1.77	0.81	3.68
Genistein 4'-sulfate-7-glucuronide (G-4'S-7G)	2.13	0.38	5.23	1.57	7.36
Genistein 4'-glucuronide (G-4'G)	2.46	0.39	2.85	0.72	5.32
Genistein 7-glucuronide (G-7G)	3.09	0.61	1.87	0.86	4.96

a) They are listed in the appearance in the sinusoidal blood in all three treatments: genistein tablet; soy milk and genistein tablet with low-fat cow milk.

b) τ , t_{peak} , and their sum t_{max} are explained in Fig. 2. The respective standard deviations (SD) from jackknife are listed. The detailed calculations are available in the Supporting Information (Table 2).

3.3 Population-based modeling revealed order of genistein phase II metabolites appearance

Fixed treatment effects, between-individual variance (Ω) and within-individual (Γ) variance were calculated in Monolix, with treatment as categorical variable. The treatment effect for the genistein tablet treatment was set to 0, resulting in the other treatments being expressed with respect to the genistein tablet treatment. Since the treatments were intermitted by washout periods no influence of treatment order was assumed. Results of population-based modeling are presented in Supporting Information Table 2 and include population estimates of τ , lagtime; V , volume of distribution; k_a , absorption rate constant; k_{el} , elimination rate constant. The effect of soy milk treatment on the volume of distribution (decreased volume) was significant in G-4'G, G-7G, and G-4'G-7S. In the jackknife models, the volume of distribution for these metabolites is consistently lower for the soy treatment. The soy milk treatment also had a significant effect on the absorption rate of G-4',7-diG.

Table 3 provides time measures, which are explained in Fig. 2. The time to reach maximum plasma concentration, t_{max} , is the sum of τ , the lag between the administration of the treatment and the appearance of the metabolite, and t_{peak} , the time between the first appearance of the metabolite and when it reaches maximum concentration. The calculated τ values in Table 3 represent their appearance of the metabolites in the plasma. The lag time order was consistent in all three treatments: G-4',7-diG < G-7S < G-4'S-7G < G-4'G < G-7G. The lag times after the soy milk treatment appeared

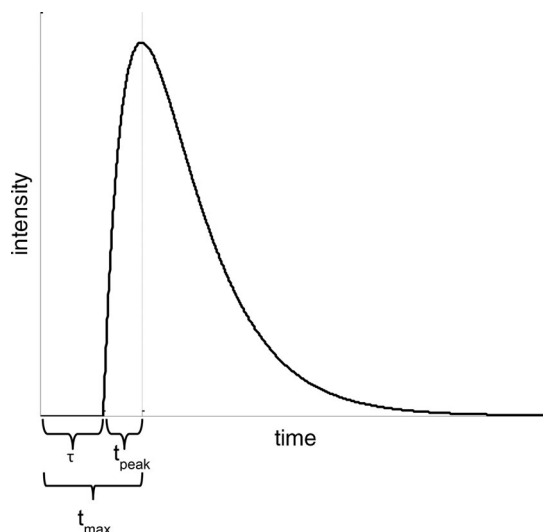


Figure 2. Explanation of the parameters τ , t_{peak} , and t_{max} used in the calculation of values presented in Table 3. τ is the time that passes between the start of the experiment and the appearance of a metabolite in the plasma and t_{peak} the time after τ that it takes to reach maximum intensity. The sum of the two, t_{max} , is the equivalent of the noncompartmental t_{max} .

generally larger than after both tablet treatments, while t_{max} were smaller.

We compared the population estimates for five phase II metabolites with their group means from the noncompartmental analysis (see Supporting Information Table 3). The

correlation coefficients for t_{\max} , AUC, and I_{\max} are all larger than 0.9, although the means from the noncompartmental analysis are generally lower than the population estimates. For $t_{1/2}$ the correlation coefficient is 0.89 (Supporting Information Table 2) and the difference between the noncompartmental means and the population estimates is more pronounced, with the noncompartmental estimates being larger. In the noncompartmental analysis, k_{el} is calculated in the elimination phase, where k_a is assumed to no longer influence the time profile. If the absorption in this phase is still significant, k_{el} is underestimated and $t_{1/2}$ is estimated too large. In the population analysis we used a one-compartment bi-exponential model and the amount of absorption in the elimination phase is taken into account in the estimation of k_{el} . This may result in more accurate estimates of absorption/elimination characteristics.

4 Discussion

Previous studies generally reported pharmacokinetic data of isoflavones by estimating the phase II metabolites after measuring the free aglycones released by enzymatic hydrolysis (for review, [38]). In more recent studies [24, 26] it was possible to assess metabolism and disposition of isoflavone-conjugated metabolites in direct measurements of the metabolites without hydrolysis. This was a major step towards understanding the influence of the chemical form of genistein on its disposition. Similar to these new reports [24, 26], our study confirmed G-7G-4'S and G-4',7-diG to be the major phase II conjugates, whereas unconjugated genistein and genistein-4'-sulfate (G-4'S) were the least abundant. We could not detect the proposed genistein-4',7-disulfate (G-4',7-diS) [39] using the LC-LTQ-Orbitrap FTMS methodology. Interestingly, to the already known metabolites G-4',7-diG, G-4'S-7G, G-7G, G-4'G, G-7S, and G-4'S, we newly identified G-4'G-7S in human plasma.

In order to enter the body the sugar moiety of the glucoside form of genistein needs to be cleaved by the microbiota by beta-glucosidases [5]. Currently, it is not clear what drives the absorption efficiency of the aglycone. In the literature, conflicting results were reported. Higher bioavailability of genistein provided as its glucoside was shown by some groups [23, 24], whereas others indicated greater bioavailability of the aglycone [40, 41] or noted no difference between the absorption of the aglycone and that of the glucoside [25, 42]. In the present study, we observed a higher absorption of soy milk genistin compared to genistein tablet independent of the food matrix (water or low fat milk). The genistein used in our study was an unformulated form, which possibly explains the lower bioavailability of the aglycone. Results from a separate study indicated that formulated genistein has comparable bioavailability to the soy milk genistin (unpublished results). Differences in formulation presumably explain the lower bioavailability of the aglycone in this study. Formulation of genistein seems therefore necessary

to achieve the optimal bioavailability for supplements or foods.

The objective of our nutrkinetic analysis was to determine the order of appearance of the phase II metabolites of genistein and the influence of food format on plasma disposition. We applied a metabolomics approach linked to nutrkinetic modeling in a cross-over randomized clinical trial. Yet, a number of human trials have been performed that define pharmacokinetic properties of genistein [23, 25, 40, 42–44] and its conjugates [24, 26, 27], but none of them applied nutrkinetics modeling for the purpose to define the order of appearance of the respective metabolites in plasma. To our knowledge the present study is the first that has successfully applied this technology. We could show that in peripheral blood, genistein supplementation independent of the food matrices followed a defined pattern: G-4',7-diG appeared first, followed by G-7S, G-4'S-7G, and G-4'G, while the G-7G always was the last. This information is of interest, since it demonstrates that phase II metabolism of genistein is highly regulated. After passing the intestine and liver, genistein appears first in a di-glucuronidated form. It took more than one hour for G-7S, and another half hour for G-7G-4'S, which is one of the major genistein metabolites, to appear. The last metabolite G-7G needed 2 hours longer than G-4',7-diG to become detectable in plasma. Yuan et al. [24] showed that G-7G clearly is the most abundant phase II metabolite in urine: the urinary excretion of G-7G was more than five times higher than the second abundant phase II metabolites (G-4'G and G-4',7-diG) in urine. It remains to speculate whether all the different genistein conjugates detected are formed de novo in the intestine and/or liver after enterohepatic circulation of genistein aglycone in an initial step, or whether the subsequent conjugates are formed in a second step after the first appearing form, G-4',7-diG, has been circulated over the body tissues. Yang et al. [16] demonstrated that within a tissue genistein glucuronides can be hydrolyzed back into genistein aglycone. In fact, deglucuronidation by cellular β -glucuronidases seems to be a common mechanism for many polyphenols [45]. If G-4',7-diG is indeed firstly distributed into peripheral tissues where genistein aglycone can be released by deglucuronidases, many possibilities remain for the formation of newly conjugated metabolites. More than 19 isoforms of UDP-glucuronosyltransferases [45–47] and more than 13 members of four sulfotransferases families [48, 49] have been identified in humans, and these isoforms have a broad and distinct tissue distribution.

Independent of the highly similar lag times (τ) of G-4',7-diG appearance in plasma in all treatments, soy milk consumption statistically significant shortened the t_{peak} of G-4',7-diG. This observation was statistically significant in both the noncompartmental and the population-based models. The genistein tablet in water or the genistein tablet in low fat milk were both similar, but showed a delayed t_{peak} and t_{\max} , respectively, when compared to soy milk. We speculate that the higher delay in case of tablet was due to the fact that in these tablets the aglycone was unformulated and thereby

less bioavailable. Alternatively, the genistein aglycone was absorbed for its largest part in the colon and not in the intestinal compartments, or encountered an enhanced enterohepatic circulation. The mechanism underlying the observed difference between tablet and soy milk in genistein nutrkinetics remains to be further explored.

The postulated formation of 5-hydroxy-equol by gut bacteria after genistein supplementation [9] could not be demonstrated in our study, which is in line with recent observations in plasma of gnotobiotic rats [7]. However, we were able to detect 5-hydroxy-equol in plasma after soy milk treatment, suggesting that this compound is a gut-derived metabolite of daidzein rather than of genistein. To resolve the question of whether 5-hydroxy-equol is produced from genistein by human microbiota, we would need to apply labeled tracers studies in healthy adults.

The two major gut-derived genistein metabolites, i.e. dihydrogenistein and 2(4-hydroxy phenyl)propionic acid, appeared in the plasma according to the order of their formation [10–12]: dihydrogenistein reached t_{\max} before 2(4-hydroxy phenyl)propionic acid. However, the inter- and intraindividual variations were very high in all treatments. The AUC of 2(4-hydroxy phenyl)propionic acid was ten times higher compared to the AUC of dihydrogenistein. The 2(4-hydroxy phenyl)propionic acid metabolite reached an AUC level comparably high to the major phase II metabolite and irrespective of the treatment, suggesting a functional enterohepatic circulation linked to an active microbiota processing. Unfortunately, for none of the gut-derived metabolites a terminal elimination half-time could be calculated, due to the high intra- and interindividual variability of the subjects. This high variability of the gut-derived metabolites could not be obviated, although we applied a housed clinical setting that controlled the timing, the amount, and the composition of the supplied meals in all treatments. In future studies, further stratification of subjects using other additional parameters (e.g. microbiota composition, genetic background) might potentially be helpful.

Novel methodologies in nutrkinetics that have been developed in the recent years allow to follow the metabolic fate of nutrients using the metabolomics approach (reviewed in [29]). The technology of nutrkinetic modeling for mechanistic considerations of metabolite function requires a crossover design to reduce confounding sources of variation, highly sensitive analytical instrumentation for simultaneous metabolomics profiling, like in the present study, and a dedicated modeling strategy. This mathematical modeling approach will become instrumental for future dietary interventions studies, where highly regulated homeostatic systems in healthy individuals will be explored [50, 51]. The present analysis nicely shows the potential of the nutrkinetics approach. With its use we could determine the order of appearance of the phase II metabolites of genistein, an important nutraceutical in human health. The nutrkinetics approach has of course its limits, as it relies on the quality of the large data sets and the underlying knowledge of the nutrdynamic processes. To the

best of our knowledge, this is the first study that applied the nutrkinetics approach for the soy isoflavone genistein.

In conclusion, we used a highly sensitive LC-LTQ-Orbitrap FTMS platform to detect genistein and its metabolites in human plasma and developed a new nutrkinetic model to determine the order of appearance of phase II metabolites. Population-based nutrkinetic modeling is a powerful tool to support the elucidation of the metabolic fate of phytonutrients in human plasma. The current study showed that genistein aglycone in water and in low-fat milk had highly similar nutrkinetic properties, i.e. no influence of the low-fat milk on the systemic exposure of phase II genistein metabolites was observed. The periods for the lag times (τ) and for the t_{\max} were highly similar and the order of the metabolites was conserved. We identified G-4'G-7S, a new phase II metabolite, in human plasma. For all three application forms (genistein tablet in water, genistein tablet in low-fat milk or soy milk genistin) the plasma disposition of the metabolites was in the following order: (1) G-4',7-diG, (2) G-7S, (3) G-4'S-7G, (4) G-4'G, (5) G-7G. Interestingly, after intake of the different genistein or genistin formats, we observed G-7G-4'S and 2(4-hydroxy phenyl)propionic acid to be the major phase II metabolite and the major gut-derived metabolite, respectively, for all formats. The double glucuronidated metabolite G-4',7-diG appeared fastest (<1 h) whereas all mono sulfated, hetero-conjugated and mono glucuronidated forms followed after a time lag of >1 h. The metabolic fate of the gut-derived genistein metabolites showed high intra and interindividual variations, which impeded population-based nutrkinetic modeling. Given the fact that the bioavailable genistein firstly appeared as a double glucuronidated conjugate (G-4',7-diG) in plasma and the subsequent genistein conjugates appear as mono- or hetero-conjugated forms, we speculate that G-4',7-diG is the primary genistein form, while the others may be derived from secondary conjugations after tissue distribution and enzymatic hydrolysis of G-4',7-diG. Future studies will need to investigate which of the secondary conjugations are generated through enterohepatic recycling and which are taking place in genistein target organs.

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