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A novel method for the quantitation of gingerol glucuronides in human plasma or urine based on stable isotope dilution assays

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ABSTRACT

The bio-active compounds of ginger (*Zingiber officinale* Roscoe), the gingerols, are gaining considerable attention due to their numerous beneficial health effects. In order to elucidate the physiological relevance of the ascribed effects their bioavailability has to be determined taking their metabolization into account. To quantitate *in vivo* generated [6]-, [8]- and [10]-gingerol glucuronides in human plasma and urine after ginger tea consumption, a simultaneous and direct liquid chromatography–tandem mass spectrometry method based on stable isotope dilution assays was established and validated. The respective references as well as the isotopically labeled substances were synthesized and characterized by mass spectrometry and NMR. Selective isolation of gingerol glucuronides from human plasma and urine by a mixed-phase anion-exchange SPE method led to recovery rates between 80.8 and 98.2%. LC–MS/MS analyses in selected reaction monitoring modus enabled a highly sensitive quantitation of gingerol glucuronides with LoQs between 3.9–9.8 nmol/L in plasma and 39.3–161.1 nmol/L in urine. The method precision in plasma and urine varied in the range ± 15%, whereas the intra-day accuracy in plasma and urine showed values between 78 and 122%. The developed method was then applied to a pilot study in which two volunteers consumed one liter ginger tea. Pharmacokinetic parameters like the maximum concentration (c_{max}), the time to reach c_{max} (t_{max}), area under the curve (AUC), elimination rate constant (k_{el}) and elimination half-life ($t_{1/2}$) were calculated from the concentration-time curve of each gingerol glucuronide. The obtained results will enable more detailed investigation of gingerol glucuronides as bioactives in their physiologically relevant concentrations.

1. Introduction

The rhizome of *Zingiber officinale* Roscoe, commonly known as ginger, is globally an important spice in food and beverages, and has been used for cuisine and medicinal purpose since ancient times. Especially, its nutraceutical properties are attracting increasing interest. The bio-active properties, including antiemetic [1], antioxidative [2], and anti-inflammatory [3–5] effects, are mainly attributed to the major pungent compounds in ginger, namely the gingerols. Gingerols are a homologous series of β-hydroxy phenolic ketones, which only differ in the length of their alkyl chain. However, several studies demonstrated a rather complex metabolization of gingerols. Oral administration of [6]-gingerol to rats led to the formation of [6]-gingerol-4′-*O*-β-glucuronide as one of the major metabolites, besides vanillic acid, ferulic acid and 9-hydroxy-[6]-gingerol [6]. Data from *in vitro* studies with NADPH-fortified rat hepatic microsomes provided evidence for eight [6]-gingerol metabolites, which were identified by GC–MS as two diastereomers of aliphatic hydroxylation and the diastereomers of two products of aromatic hydroxylation and two diastereomers of [6]-gingerdiols [7]. Further, it was shown that incubation of human UDPGA-fortified hepatic microsomes with [6]-gingerol led mainly to the biosynthesis of the respective aryl-*O*-glucuronides and small amounts of alkylic-*O*-glucuronides, whereas incubation of microsomes from intestine with [6]-gingerol resulted in the formation of aryl-*O*-glucuronides only. Both glucuronides were analyzed by means of LC–MS/MS [7]. Quantitative data on the bioavailability of gingerols in rat were obtained via simultaneous determination of [6]-, [8]- and [10]-gingerol in plasma after ingestion of ginger oleoresin via HPLC–MS [8].

Abbreviations: %CV, coefficient of variation; c_{max}, maximal plasma concentration; t_{max}, the time to reach c_{max}; AUC, area under the curve; k_{el}, terminal elimination rate constant; $t_{1/2}$, elimination half-life; SIM, selected-ion monitoring modus; HSQC, heteronuclear single quantum coherence; HMBC, heteronuclear multiple bond coherence; NADPH, reduced form of nicotinamide adenine dinucleotide phosphate; SIDA, stable isotope dilution assays; UDPGA, uridin-5′-diphosphoglucuronic acid.

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However, up to now, studies on the metabolic fate of gingerols in humans are still rare. Zick et al. [9] demonstrated that after administration of 100 mg–2.0 g ginger extract to 27 healthy volunteers neither non-metabolized [6]-, nor [8]-, nor [10]-gingerol were detectable in human plasma using an HPLC electrochemical detection method. Regarding the respective metabolites, gingerol glucuronides were detected after oral intake of 1.0 g ginger extract. Limits of quantitation for gingerols were determined as 0.1 μg/mL for [6]- and [8]-gingerol, and 0.25 μg/mL for [10]-gingerol [9]. Recently, a more sensitive LC–MS/MS(ESI) method was developed to determine plasma pharmacokinetics of gingerols in humans after ginger extract ingestion [10]. In this study, the LoQs of all gingerols were calculated at 5 ng/mL.

However, it has to be noted that none of the described methods allow for direct quantitation of gingerol glucuronides probably due to the absence of the respective reference substances. Instead, the concentration of gingerol glucuronides was calculated as the difference of gingerol concentrations prior and after β-glucuronidase hydrolyzation.

To evaluate a potential bioactivity of gingerols their metabolism has to be taken into account, which initially requires sensitive and direct quantitation of the major metabolites. Thus, a LC–MS/MS-method based on stable isotope dilution assays was developed and validated, including the synthesis of the respective reference as well as the isotopically labeled substances. This newly developed quantitation method was then applied to a small-scale pilot study in order to determine pharmacokinetic parameters of gingerols in humans after consumption of ginger tea.

2. Materials and methods

2.1. Chemicals

Unless otherwise stated, all chemicals were obtained from Sigma-Aldrich (Taufkirchen, Germany). Solvents used as mobile phases in liquid chromatography were from Merck Millipore (Merck Chemicals, Darmstadt, Germany).

2.2. Synthesis of unlabeled and labeled gingerol glucuronides

For the synthesis of unlabeled and labeled gingerol glucuronides two equivalents sodium methylate in 5 mL methanol were stirred under an argon atmosphere, and a solution of one equivalent of the respective gingerol ([6]-gingerol 0.87 mmol; [8]-gingerol: 0.14 mmol; [10]-gingerol: 0.11 mmol) or [$^2H_4$]-gingerol ([$^2H_4$-6]-gingerol: 0.67 mmol; [$^2H_4$-8]-gingerol: 0.31 mmol; [$^2H_4$-10]-gingerol: 0.04 mmol) in anhydrous methanol (0.08 mmol/mL) was added dropwise. [$^2H_4$]-gingerols were synthesized as described recently [11]. After stirring for 3 h, two equivalents of acetobromo-α-D-glucuronic acid methyl ester in methanol were added dropwise at 40 °C [12]. The mixture was stirred for another 3 h, before it was cooled to RT and adjusted to pH 8 with sodium carbonate (0.1 mol/L). The solvent was distilled off under vacuum (40 °C, 300 mbar) and the residue was redissolved in 15 mL ammonium formiate buffer (0.1 mol/L, pH 8.2) and washed three times with ethyl acetate. In case of unlabeled and labeled [6]-gingerol glucuronide, no contaminations occurred in the aqueous phase. Unlabeled and labeled [8]- and [10]-gingerol glucuronides were isolated from contaminants by semi-preparative HPLC-UV at 280 nm with a flow rate of 3 mL/min. Here, a Phenomenex Hyper-Clone C18 (250 × 10 mm, 5 μm, 80 Å, Aschaffenburg, Germany) was used as stationary phase, the mobile phase A and B are comprised of ammonium formiate buffer (0.1 mol/L, pH 8.2) and methanol.

2.3. Characterization of gingerol glucuronides by mass spectrometry and nuclear magnetic resonance spectroscopy (NMR)

For the characterization of the synthesized gingerol glucuronides their specific masses were analyzed by means of one-dimensional mass spectrometry in negative electrospray ionization mode using a Finnigan MAT ion trap LCQ-MS (Bremen, Germany). The used ion trap parameters were as follows: source voltage: 5 kV, source current: 80 μA, sheath gas flow: 60 arbitrary units, aux gas flow: 5 arbitrary units, capillary temperature: 200 °C, capillary voltage: −10 V.

In case of unlabeled [6]-gingerol glucuronide the synthesis yield was high enough for NMR analysis. ^{1}H-, ^{13}C NMR, COSY, HSQC and HMBC spectroscopy analysis were performed by means of a Bruker Avance III (500 MHz, 300 K, Rheinstetten, Germany). [6]-gingerol glucuronide was dissolved in D_2O. Data processing was performed using Topspin version 1.3 (Bruker, Rheinstetten) and MestReNova software (Mestrelab Research, Santiago de Compostela, Spain). The spectroscopic data were as follows:

[6]-gingerol glucuronide (**1**, Fig. 1A) LC–MS (ESI$^-$): *m/z* 469 [M−1]$^-$. ^{1}H NMR (500 Hz; D_2O; 300 K): δ 7.04 (d, 1H, *J* = 8.2, H-$C_{5'}$), 6.91 (s, 1H, H-$C_{2'}$), 6.78 (d, 1H, *J* = 7.7, H-$C_{6'}$), 5.03 (dd, 1H, *J* = 13.7; 5,6, H-$C_{1''}$), 3.97 (m, 1H, H-C_5), 3.81 (s, 3H, H-$C_{7'}$), 3.77 (m, 1H, H-$C_{5''}$), 3.54 (d, 3H, *J* = 8.2, H-$C_{2''-4''}$), 2.85 (d, 2H, *J* = 12.0; 5.5, H-C_2), 2.81 (d, 2H, H-C_1), 2.58 (m, 2H, H-C_4), 1.26 (m, 8H, H-C_{6-9}), 0.78 (t, 3H, H-C_{10}). ^{13}C NMR (500 Hz, D_2O, 300 K): δ 215.3 (C_3), 181.0 ($C_{6''}$), 148.6 ($C_{3'}$), 143.7 ($C_{4'}$), 136.8 ($C_{1'}$), 120.9 ($C_{6'}$), 116.5 ($C_{5'}$), 113.1 ($C_{2'}$), 100.6 ($C_{1''}$), 75.4 ($C_{5''}$), 72.7 ($C_{3''}$), 71.7 ($C_{4''}$), 67.7 (C_5), 57.2 ($C_{2''}$), 55.9 ($C_{7'}$), 49.7 (C_4), 44.2 (C_2), 36.0 (C_6), 28.7 (C_1), 24.1 (C_7), 21.8 (C_9), 13.3 (C_{10}). **[8]-gingerol glucuronide** (**2**, Fig. 1B): LC–MS (ESI$^-$): *m/z* 497 [M−1]$^-$. **[10]-gingerol glucuronide** (**3**, Fig. 1C): LC–MS(ESI$^-$) *m/z* 525 [M−1]$^-$. **[$^2H_4$]-[6]-gingerol glucuronide** ([$^2H_4$]-**1**, Fig. 2A): LC–MS(ESI$^-$) *m/z* 473 [M−1]$^-$. **[$^2H_4$]-[8]-gingerol glucuronide** ([$^2H_4$]-**2**, Fig. 2B): LC–MS(ESI$^-$) *m/z* 501 [M−1]$^-$. **[$^2H_4$]-[10]-gingerol glucuronide** ([$^2H_4$]-**3**, Fig. 2C): LC-MS (ESI$^-$) *m/z* 529 [M−1]$^-$.

2.4. Development of stable isotope dilution assays (SIDA) for the direct quantitation of gingerol glucuronides by means of LC–MS/MS

2.4.1. Human pilot study

The pilot study was conducted as described recently [11]. Briefly, each of two female volunteers consumed one liter ginger tea, prepared of 100 g fresh, chopped Chinese ginger rhizome. Blood samples were taken at baseline, 30, 60, 90 and 120 min after ginger tea intake. Thus, the study volunteers consumed 96.6 μmol of [6]-gingerol, 4.4 μmol of [8]-gingerol and 2.2 μmol of [10]-gingerol within 20 min. The plasma was separated immediately from erythrocytes by centrifugation at 4 °C (1950 x *g*, 15 min) and stored at −80 °C until usage. Urine samples were collected from one volunteer at baseline, as well as in different time periods between 1 and 3 h, 3–6 h, 6–9 h, 9–12 h and 12–24 h after ginger tea consumption. The urine was stored at −20 °C until usage.

All analyses were in accordance with the Helsinki Declaration of 1975, as revised in 2013. Since two of the authors (C.S. and G.A.) volunteered as subjects, no informed consent documents had to be signed.

2.4.2. Preparation of plasma and urine samples for LC–MS/MS analysis

For the determination of gingerol glucuronide concentrations in plasma 0.05 nmol **[$^2H_4$]-1**, 0.05 nmol **[$^2H_4$]-2** and 0.02 nmol **[$^2H_4$]-3** were added to 1 mL plasma. Urine (1 mL) was spiked with 1.4 nmol **[$^2H_4$]-1**, 0.19 nmol **[$^2H_4$]-2** and 0.05 nmol **[$^2H_4$]-3** for