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A strategy for absolute quantitation of isomers using high performance liquid chromatography-ion mobility mass spectrometry and material balance principle

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ABSTRACT

Accurate characterization of isomers is a quite challenge and time-consuming work. A major bottleneck is the lack of relevant reference standards. In this work, we conducted a proof-of-concept study to develop a standard-free method for absolute quantitation of isomers by using high performance liquid chromatography-ion mobility mass spectrometry and material balance principle. The isomer structures were characterized by matching the rank order of experimental collision cross sections (CCSs) to that of theoretical CCSs. Then a time-dependent hydrolysis protocol derived from material balance equation was used for the calculation of the relative correction factor (*F*). Finally, the multi-level normalized matrix approach was developed to absolutely and precisely quantitate isomeric compounds. To assess this method, quercetin-liver microsome reaction pool was applied. With the developed method, four isomeric metabolites of quercetin were accurately analyzed, and metabolic profiling of quercetin isomeric metabolites was firstly reported with the absolute quantitation results. This is an accurate yet simple method for the absolute quantitation of positional isomers of interest.

1. Introduction

Interests in the separation, identification, and quantitation of isomers are largely motivated by its unique chemical and biological properties. For example, different perfluoro octane sulfonate (PFOS) isomers are associated with birth weight, increase of the branched PFOS lead to a reduction in the weight of infants at birth, and linear PFOS has little impacts [1]. What's more, lyso-Gb3 a novel biomarker of Fabry disease is significantly abundant than its isomers in plasma, which presents the opposite case in urine [2]. In aspect of analytical view, there remains a challenging task for qualifying and quantitating isomers due to their highly similar structure and generally the absence of reference standards [3]. Therefore, developing methods for characterizing isomers has always been a demanding, challenging, and significant work for various scientific fields.

Many analysis techniques have been developed to work out this issue such as hyphenated mass spectrometric with chromatographic techniques [4–7] and nuclear magnetic resonance [8–10]. Moreover, various derivatization methods and special interface materials are frequently used to distinguish the isomers, *e.g.* methylation to phospholipids [11], tetrabutylammonium hydroxide to PFOS [1] and solid-phase permethylation of glycans [12]. Most recently, ion mobility mass spectrometry (IMS), a combination of ion mobility spectrometry and mass spectrometry, has become a powerful multi-dimensional separation technique [13,14]. It can provide an additional dimension information namely gas-phase collision cross section (CCS) for the structural identification of isomers [15,16]. Except for the enantiomers, the IMS systems could separate the isomers theoretically when their space configurations and binding sites are different, reflecting on the difference of CCS value [17].

Although abovementioned methods have been applied for structural identification of isomers, quantitative analysis of isomers is often impeded due to the absence of reference standards. The quantitation of isomers is paramount of importance in chemical and biological analysis, as well as for the study of metabolism [18,19]. For example, glucuronidation a common phase II metabolism is a well-known reaction by the addition of glucuronide to a substrate, resulting in the generation of isomeric metabolites with different bioactivity [20–22]. As previously reported, glucuronidation of estradiol will produce two

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isomeric products *i.e.* estradiol-3-O- and 17-O-glucuronidation, which present quite different physiological role in metabolism [23].

Therefore, in this work we proposed an absolute quantitation method based on the differences in CCSs and material balance (MB) for the analysis of positional isomers derived from one parent compound. In a fixed reaction pool, the dynamic equilibrium can be described as the amount of each isomer is changing constantly, but the total molar number of isomers keeps constant [24]. As shown in Fig. 1, after the separation of these four isomeric metabolites based on the different chromatographic behaviors, structural identification of isomeric products is firstly performed by IMS *via* the matching of experimental CCS (ECCS) and theoretical CCS (TCCS). Thereafter, isomeric products are program hydrolyzed, and the peak area of individual metabolite at each time point are used for the calculation of the relative response factors (*F*). In the last step, normalized *F* value is used for quantitative analysis of isomeric products without requirement of the reference standards. In this method, calculation of *F* is not restricted by the hydrolysis ratio of the isomers, which means whether the metabolites can be hydrolyzed completely or not will has no effect on the calculation. With this developed method, the accurate amount of four isomeric metabolites of quercetin produced by rat liver microsomes were determined, and their dynamic formation processes were unraveled for the first time.

2. Experimental

2.1. Chemicals and reagents

Quercetin and liquiritigenin (internal standard, IS) were purchased from National Institute for the Control of Pharmaceutical and Biological Product (Beijing, China). Quercetin-3-O-β-D-glucuronide (Q-3-G) was purchased from Zzbio (Shanghai, China). Quercetin-4′-O-β-D-glucuronide (Q-4′-G), quercetin-7-O-β-D-glucuronide (Q-7-G) and quercetin-3′-O-β-D-glucuronide (Q-3′-G) was prepared in our lab. The Sprague Dawley (SD) rat liver microsomes were purchased from RILD (research institute for liver diseases, Shanghai, China). The β-glucuronidase from Helix pomatia (Type HP-2, aqueous solution, ≥100,000 units/mL), saccharic acid 1,4-lactone, uridine diphosphate glucuronic acid (UDPGA) and other chemicals used were purchased from Sigma-Aldrich (Gillingham, UK). Methanol (LC–MS grade) was purchased from Merck (Darmstadt, Germany).

2.2. In vitro rat liver microsomal incubation

The typical reaction mixture (200 μL) was composed of 100 mM potassium phosphate buffer (pH 7.4), 5 mM $MgCl_2$, 2 mM UDPGA, 1 mM saccharic acid 1,4-lactone, 50 μM quercetin and 5 μL liver microsomes (protein concentration remained 0.5 mg/mL). After 5 min preincubation at 37 °C, the reaction was initiated by the addition of 2 mM UDPGA. All reactions were incubated at 37 °C for 10–60 min, and terminated by adding 300 μL ice-cooled methanol. The residual was dried under N_2 and redissolved with 200 μL methanol. After centrifuge, the supernatants were analyzed by LC–MS.

2.3. Experimental CCSs measurement of isomers

An Agilent 1290 rapid resolution system equipped with a 6560 ion mobility Q-TOF mass spectrometer containing an electrospray ionization source (Agilent Technologies, CA, USA) was utilized for the measurement of CCS of isomers. The separations were obtained on an Agilent ZORBAX Eclipse Plus-C18 column (2.1 mm × 50 mm, 1.8 μm) at 30 °C with the mobile phase solvent 0.1% formic acid in H_2O (A) and MeOH (B). The ESI source worked in the negative ion mode. Instrumental settings of MS were optimized as follows: drying gas temperature, 325 °C; drying gas flow rate, 7 L/min; nebulizer pressure, 45 psi; sheath gas temperature, 350 °C; sheath gas flow rate, 11 L/min, capillary voltage, 4000 V; nozzle voltage, 2000 V; HP pressure funnel RF, 200 V; Trap funnel RF, 200 V; IM-real funnel RF 150 V; collision gas, N_2. The MS data were analyzed using Browser for MassHunter IMS Acquisition Data software (Agilent Technologies, CA, USA).

2.4. Calculation of theoretical CCSs

The TCCS was calculated by the workflow named 1st G (first generation), which was performed by a service company (http://ccscalc.com/index.html).

2.5. Quantitative LC-MS/MS analysis

Quercetin metabolites were dissolved in methanol, and a 100 μL sample reacted with 5 μL β-glucuronidase for hydrolyzing under pH 5 at 37 °C for 6 h. The hydrolysis samples were detected by LC–MS every 15 min A Shimadzu LC-30 A ultra-fast liquid chromatography system equipped with an 8050 triple quadrupole mass spectrometer (LC-QQQ MS) was used for quantitative analysis of hydrolysis samples. Separations were carried out at 30 °C on an Agilent ZORBAX Eclipse Plus-C18 column (2.1 mm × 50 mm, 1.8 μm) with the mobile phase consisting of 0.1% formic acid in H_2O (A)-MeOH (B). The flow rate was 0.2 mL/min. The injection volume was 5 μL. The step gradient was set as the following program: 0–2 min, 30–45% B; 2–5 min, 45% B; 5–9 min, 45–60% B; 9–12 min, 60–90% B; 12–16 min, 90–100% B; 16–18 min, 100% B. The acquisition conditions were as follows: gas temperature, 300 °C, dry gas flow rate, 10 L/min, nebulizing gas flow rate, 3 L/min, heating gas flow rate, 10 L/min, dwell time: 28 ms for each ion pair. Data were processed in the Quant Browser Window of the workstation offline.

2.6. Calculation of relative response factors (F)

According to MB, Eq. (1)–(3) was deduced for calculation of *F*.

$$\sum(A_{Mn}/a_{Mn}) + A_P/a_P = P_0(n = 1\text{to}4) \quad (1)$$

Where P_0 is the total molar number of feeding quercetin. $\boldsymbol{A}_{Mn}$ (n = 1 to 4) and $\boldsymbol{A}_P$ are the detected peak area of individual isomeric metabolite and quercetin, respectively, which could be obtained from the instrument and are known; $\boldsymbol{a}_{Mn}$ (n = 1 to 4) and $\boldsymbol{a}_P$ are peak area per mole of individual isomeric metabolite and quercetin, respectively, which are unknown and could be calculated from the group of five-element equations. All peak area of analytes is normalized with peak area of IS.

$\boldsymbol{A}$/ $\boldsymbol{a}$ stands for "detected area / (area per mole)" which is just the molar number of the four individual metabolites and prototype quercetin in this reaction pool, respectively. Thus, the sum of $\boldsymbol{A}$ / $\boldsymbol{a}$ should be equal to P_0. The hydrolyzed sample was detected per quarter from 0 h to 6 h, and totally 25 groups hydrolysis data were obtained. Five groups of data were randomly selected from 25 groups, making a matrix (5 × 5) which contained five variables (M1, M2, M3, M4 and hydrolyzed quercetin). Thus, the unknown $\boldsymbol{a}_{Mn}$ (n = 1 to 4) and $\boldsymbol{a}_P$ could be routine calculated.

The relative response factor F_n of each metabolite (n = 1 to 4) is calculated by area per gram of the metabolite to that of quercetin and used for the absolute quantitation of isomeric metabolites. The F_n of individual metabolite can be calculated by Eq. (2),

$$F_n = (a_{Mn}/m_n)/(a_P/m_P)(n = 1\text{to}4) \quad (2)$$

Where $\boldsymbol{a}_{Mn}$ (n = 1∼4) and $\boldsymbol{a}_P$ are peak area per mole of individual isomeric metabolite and quercetin, which were obtained

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