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A simple LC–MS/MS method for the simultaneous quantification of resveratrol and its major phase II metabolites: Assessment of their urinary and biliary excretions in rats

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ABSTRACT

Trans-resveratrol (Res) is rapidly metabolized, extensively distributed into various tissues and mainly excreted by urine. The present study aimed to establish a simple LC–MS/MS method to simultaneously quantify Res and its major phase II metabolites (Res-3-*O*-β-D-glucuronide, R3G; Res-4′-*O*-β-D-glucuronide, R4′G; Res-resveratrol 3-sulfate, R3S; and Res-4′-sulfate, R4′S), and apply this method to assess their urinary and biliary excretions in rats. A simplified salting-out assisted liquid–liquid extraction (SALLE) strategy was developed to prepare samples with acetonitrile-methanol mixture (8:2, v/v) as extractant and ammonium acetate solution (10 M) as salting-out reagent. The method validation demonstrated an acceptable recovery (>80%), good accuracy (85–115%), low deviation of detection (<15%) and no obvious matrix effect (<20%). Then the validated method was successfully applied to analyze the excretion of Res and its metabolites after intragastric administration of Res at 50 mg/kg in rats. Only a minor proportion of Res (0.51 nmol) and its metabolites (R3S, 35.8 nmol; R4S, 0.25 nmol; R3G, 142.3 nmol; R4′G, 0.19 nmol) were eliminated via bile, while the majority of Res (1670.2 nmol), R3G (14,089.0 nmol) and R3S (2975.6 nmol) were excreted through urine. The major forms found in feces were Res and R3S, which were accumulated up to 241.8 and 250.8 nmol, respectively. In summary, the SALLE technique simplified the samples preparation and could be well popularized, especially for those highly polar compounds in biosamples like urine, bile and feces, where various endogenous substances could significantly affect the extraction recovery and detection response.

1. Introduction

Trans-resveratrol (Res), a renowned polyphenol compound has been substantially reported for its various health protection and diseases alleviation functions, such as antioxidant, cancer prevention, anti-inflammatory and anti-aging [1,2]. However, orally administered Res undergoes extensive metabolism before reaching the circulation system and other tissues, which mainly generates the glucuronide and sulfate conjugates with higher plasma concentrations than their parent compound. Hence, the fast first-pass metabolism of Res significantly contributes to its low oral bioavailability, which was reported up to 30% in rats [3,4]. Numerous references have elaborately reported the metabolism, excretion and tissue distribution of Res [5]. Monitoring the amounts of Res and its metabolites not only in urine but also in bile and feces would help us better understand the disparity between the pharmacokinetic behavior and its pharmacological response, so as to demonstrate the reason why it exhibited health benefits with such low bioavailability.

Among its metabolites, *trans*-resveratrol 3-*O*-β-D-glucuronide (R3G) and *trans*-resveratrol 4′-*O*-β-D-glucuronide (R4′G) are two representative mono-glucuronide conjugates, *trans*-resveratrol 3-Sulfate (R3S) and *trans*-resveratrol 4′-sulfate (R4′S) are two prominent mono-sulfate conjugates in our present study. Several methods have been developed to analyze Res and its metabolites in urine, bile or feces. Nonetheless, the majority of them have ignored R4′S, which is a potential beneficial metabolite [6]. These reported methods mainly applied HPLC, LC–MS and LC–MS/MS with the solid phase extraction (SPE) or direct protein precipita-

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tion (PPT) as sample preparation procedure [7,8]. Some methods required the in vitro glucuronidase or sulfatase hydrolysis to generate Res in order to indirectly determine its conjugates, and some other analytes may be not cost-effective due to time-consuming and laborious procedures, large sample volumes or matrix interference. For example, the components of bile salts, cholesterol and phospholipids contained in bile can cause significant matrix interferences. The majority of urine is water (above 95%), and the feces are mainly emerged as solid form. Traditional preparation methods could not guarantee the accuracy and recovery of target analytes in these biological samples. While SPE shows remarkable advantages over the conventional extraction methods, including shorter extraction time, lower solvent volume but higher extraction recovery, and wide application. However, apart from the high cost, SPE still needs multiple procedures (column activation, elution, loading, collection, evaporation and reconstitution).

In this regard, we tried to establish a simplified sample preparation method to simultaneously quantify the amounts of Res, R3G, R4′G, R3S and R4′S in rat urine, bile and feces. Hereby, we resorted to a salting-out assisted liquid–liquid extraction (SALLE) strategy [9]. In this method, an acetonitrile-methanol mixture was firstly used as the organic phase to extract the target analytes, followed by the addition of ammonium acetate buffer (10 M) as salting-out reagent. The present method was validated on its precision, matrix effect, extraction recovery and stability under different conditions (three freeze-thaw circles, storage at 4 °C for 24 h post-preparation, storage at −70 °C for 15 days). Afterwards, the validated method was applied to profile the excretion of Res, R3G, R4′G, R3S and R4′S in urine, bile and feces.

2. Materials and methods

2.1. Chemicals and reagents

Res (purity, 98%) and its merchandized metabolites R3G (purity, 98%), R4′G (purity, 95%), R3S (purity, 99%) and R4′S (purity, 98%) were all purchased from J&K Chemical Ltd. (Shanghai, China). The internal standard (ISTD) wogonin (98%) was kindly offered by the Department of Physiology, China Pharmaceutical University (Nanjing, China). The methanol and acetonitrile (HPLC grade) were purchased from Tedia (Fairfield, OH, USA). The sodium carboxyl methyl cellulose (CMC-Na) and ammonium acetate were supplied by Sinopharm Chemical Reagent Co., Ltd (Shanghai, China). All the other chemicals and agents were of analytical grade and were purchased from Nanjing Chemical Reagent No.1 Factory (Nanjing, China).

2.2. Preparation of standard and quality control samples

The standard stock solution of Res and its metabolites were dissolved in methanol at 1.0 mg/mL, and stored at −20 °C. The analytes were stepwisely diluted to the target concentrations. Res, R3S, R4′S were prepared from 2000 to 4 ng/mL, while R3G was set from 10,000 to 20 ng/mL and R4′G was from 4000 to 8 ng/mL. The concentrations of quality control samples (QC, low, middle, high) were set at 10, 100, 1600 ng/mL for Res, R3S and R4′S; 50, 500, 8000 ng/mL for R3G; 20, 200, 3200 ng/mL for R4′G. The lower limit of quantitation (LLOQ) was set at 4 ng/mL for Res, R3S and R4′S, 8 ng/mL for R4′G and 20 ng/mL for R3G, respectively. The calibration samples and QC samples were prepared as follows: 50 μL working solution was spiked in 50 μL bile, urine or feces homogenate, followed by the addition of 100 μL acetonitrile-methanol mixture (8:2, v/v) containing 50 ng/mL ISTD, and then an aliquot of 10 μL ascorbic acid (VC, 10%, v/v) was added to stabilize the analytes. Eventually, 50 μL ammonium acetate buffer (10 M) was added into the mixtures. The prepared samples were vortexed for 5 min and centrifuged at 12,000 rpm for 5 min at 4 °C. An aliquot of 10 μL of the supernatant was directly injected into the LC–MS/MS system.

2.3. LC–MS/MS conditions

The processed samples were analyzed by LC–MS/MS (Shimadzu 20 AD HPLC system and TSQ mass spectrometer with ESI interface). The samples were eluted on a Shimadzu GL column (ODS-3, 2.1 × 150 mm, 5 mm). The flow rate was set at 0.35 mL/min, and the column temperature was at 30 °C. The analytes of interest were eluted by gradient elution with water (5 mM ammonium acetate) as aqueous phase (designated as phase A) and acetonitrile as organic phase (designated as phase B), depicted as follows: phase B was maintained at 10% for 0.5 min, followed by progressively augment from 10% to 50% in 7.0 min, then kept at 50% for 1 min, afterwards, it was gradually decreased to 10% in 0.5 min, and finally maintained at 10% till the end of analysis. The total run time was 11.5 min.

Quantification of target analytes was performed in SRM mode operating in negative mode. The dwell time for each transition was 0.2 s. The scan parameters were set as follows: spray voltage, −3000 v; capillary temperature, 350 °C; sheath and auxiliary gas, 35 and 15 Arb; collision argon gas, 1.5 mTorr. The parent ions of Res, R3S (R4′S), R3G (R4′G) were monitored at *m*/*z* 227.0, 306.9, 402.9 with product ions at *m*/*z* 185.0, 226.9, 227.0 under the collision energy at 24, 31, 23 eV, respectively. And the ISTD was monitored at *m*/*z* 283/268. All these data were analyzed by Xcalibur software (version 2.2).

2.4. Method validations

The determination method was validated based on the protocol in our lab, which mainly focused on the accuracy, precision, matrix effect, extraction recovery and stability. The accuracy and precision were calculated at four levels, including LLOQ and three QC levels. The extraction recovery was calculated as the ratio of the target analyte peak area from the bile/urine/feces samples to the average peak area from the extracted blank matrix spiked with Res and its metabolites at the same levels. Inter-batch precision was analyzed within three successive batches. The stability of target analytes was evaluated as follows: (a) post-preparation for 24 h at 4 °C; (b) storage at −70 °C for 15 days; and (c) three freeze-thaw circles (freezed at −70 °C and thawed at ambient temperature).

2.5. Application of the validated method to profile biliary, urinary, and fecal excretion of Res and its metabolites in rats

Male Sprague-Dawley (SD) rats were supplied by SIPPR/BK Experimental Animal Co., Ltd (Shanghai, China). The rats were kept in a humanized environment, with the temperature maintained at 20 ± 2 °C and relative humidity at 50 ± 10%. During the acclimatization period, the rats were fed with standard diet and water. Ahead of experiments, the rats were fasted overnight but with free access to water. The rats were divided into two groups with 6 rats in each group. One was for biliary excretion and the other group was for urinary excretion investigation.

For the urinary excretion study, each rat was kept in a separate metabolic cage supplied with water and food, and individually accommodated for 3 days prior to the drug administration. After overnight fast, the rats kept in cages were intragastrically administrated with Res at 50 mg/kg suspended by CMC-Na. The urine and feces were collected into separate containers at 4, 8, 12, 24, 36 and 48 h after drug administration. The samples were stored at −70 °C till analysis. Initially, the urine samples were filtered through a millipore filter (inner diameter, 0.2 μm) to remove the food debris and other granular materials. After measuring of urine volume at dif-