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Stereoselective pharmacokinetic and metabolism studies of 20(S)- and 20(R)-ginsenoside Rg_3 epimers in rat plasma by liquid chromatography-electrospray ionization mass spectrometry

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ABSTRACT

20(S)- and 20(R)-ginsenoside Rg_3 are a pair of epimers which could be deglycosylated to ginsenoside Rh_2 and protopanaxadiol (PPD) *in vivo*. To better understand the differences of pharmacokinetic parameters and metabolism behaviors of Rg_3 epimers in rat plasma, a sensitive and specific liquid chromatography coupled with tandem mass spectrometry (LC–MS/MS) method was developed and fully validated. This chromatographic method separate 20(S)-/20(R)-Rg_3, 20(S)-/20(R)-Rh_2 and 20(S)-/20(R)-PPD by gradient elution of 10 mM ammonium acetate solution (pH 5.0) and acetonitrile on a C18 column with a total run time of 15 min. 20(S)-protopanaxatriol (PPT) was used as internal standard, and multiple reaction monitoring (MRM) mode with negative electrospray ionization were performed. The lower limit of quantitations (LLOQs) were between 4.2 and 4.8 ng/ml, and the accuracies were between 91.7% and 112.2% with intra- and inter-day precisions less than 11.6%. This method was successfully applied to a pharmacokinetic study of intravenous and intra-gastric administration of 20(S)-Rg_3 and 20(R)-Rg_3 to rats. It has been found that both epimers can be deglycosylated to their corresponding chiral metabolites, *i.e.*, Rh_2 and PPD, with different extents. However, 20(R)-Rg_3 underwent single direction chiral inversion to 20(S)-Rg_3 in rats. Stereoselective pharmacokinetic parameters, metabolic degrees and chiral inversion extents of Rg_3 epimers in rats were also discussed for the first time.

1. Introduction

Panax notoginseng possesses dammarane-type tetracyclic triterpenoid ginsenosides as its saponin content exclusively [1]. Among these saponins, ginsenoside Rg_3 is a potent compound which executes a wide range of pharmacological effects, including anti-inflammatory [2], antioxidant [3], anti-diabetic [4], and neuroprotective [5] activities, etc. Moreover, ginsenoside Rg_3 is proved to be a potent anticancer agent in the treatment of lung [6], breast [7], hepatic [8], pancreatic [9] cancer and melanoma [10] etc.

The molecule of ginsenoside Rg_3 bears the chiral carbon at C-20 in its structural skeleton, thus forms a pair of optical epimers, *i.e.*, 20(S)- and 20(R)-Rg_3. The pharmacological actions of the mixture of Rg_3 epimers have been studied in the early investigation stages [11]. Nevertheless, an increasing number of researches have demonstrated a diversity of stereoselective activities between Rg_3 epimers, such as the effects on cardiovascular system [12–14], immune system [15], as well as anti-tumor [16–20], anti-oxidant [21] and antidiabetic activities [22,23]. Although the underlying mechanism of most of these stereoselective functions were still unclear, it has been mentioned that the stereo-structures of the

Abbreviations: AUC, area under the curve; C_{max}, peak plasma concentration; ig, intra-gastric; IS, internal standard; iv, intravenous; LC–MS/MS, liquid chromatography coupled with tandem mass spectrometry; LLOQ, lower limit of quantitation; MRM, multiple reaction monitoring; PK, pharmacokinetic; QC, quality control; QCL, quality control low level; QCM, quality control medium level; QCH, quality control high level; $t_{1/2}$, half-life time; T_{max}, time to peak plasma concentration; 20(S)-Rg_3, 20(S)-ginsenoside Rg_3; 20(S)-Rh_2, 20(S)-ginsenoside Rh_2; 20(S)-PPD, 20(S)-protopanaxadiol; 20(S)-PPT, 20(S)-protopanaxatriol; 20(R)-Rg_3, 20(R)-ginsenoside Rg_3; 20(R)-Rh_2, 20(R)-ginsenoside Rh_2; 20(R)-PPD, 20(R)-protopanaxadiol.

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alkane chains and hydroxyl groups on C-20 of Rg_3 epimers may cause different binding affinities to receptors [16,24,25].

Pharmacokinetic studies of 20(S)-Rg_3 and 20(R)-Rg_3 have been performed in rat [26–28], dog [29], and human plasma [30]. Some researches have also focused on the *in-vivo* or *in-vitro* deglycosylation steps from Rg_3 to Rh_2 and PPD (Fig. 1), two pharmacologically more potent saponins, which have been regarded as the major metabolic products of Rg_3 *in vivo* [26,27,31]. Unfortunately, the available literatures could not provide more information on stereoselective pharmacokinetic and metabolic processes. Firstly, most of the studies simply devoted to only one chiral conformation of Rg_3. Secondly, since the administration dosages and animal models in the literatures were not the same, it is quite difficult to compare the pharmacokinetic parameters and metabolic results between 20(S)-Rg_3 and 20(R)-Rg_3. Thirdly, very little data has been collected in previous literatures for the ig administration of Rg_3 epimers because of their low absorption and high LLOQ values.

Bae et al. [32,33] established a LC–MS/MS method for a simultaneous determination of Rg_3 and Rh_2 epimers in rat plasma, and investigated the oral administration of BST204, a purified ginseng dry extract containing equal amount of Rg_3 epimers and equal amount of Rh_2 epimers. Results showed that 20(R)-Rg_3 and 20(R)-Rh_2 were hardly detectable in rat plasma after the oral administration, however, their 20(S)-epimers both exhibited significantly higher plasma concentrations and AUCs. Although these two studies have been by far the only two studies on the chromatographic peak separation of Rg_3 and Rh_2 epimers in PK studies, they did not mention the chiral inversion and chiral metabolism in rat plasma. What have not been elucidated so far are: (1) whether 20(S)- and 20(R)-Rg_3 will be inverted to the other chiral conformation in vivo, and what are the chiral inversion ratios of these two epimers; (2) to what extent do these two epimers metabolize to their corresponding deglycosylated saponins with the same chiral conformation.

To sum up, the PK studies of stereospecific properties of Rg_3 may provide an experimental basis to explain the different pharmacological activities of these two epimers. However, the stereospecific PK researches of Rg_3 epimers have not been extensively studied so far. Thus, we developed a sensitive and selective HPLC-ESI-MS/MS method for the simultaneous determination of 20(S)-/20(R)-Rg_3, 20(S)-/20(R)-Rh_2 and 20(S)-/20(R)-PPD, in rat plasma. This method was then applied to a PK study of 20(S)-Rg_3 and 20(R)-Rg_3 both after iv and ig administrations. The differences of PK parameters, the extents of chiral inversion, and the degrees of metabolizing processes of these two epimers in rat plasma after two administration routes were comprehensively estimated and extensively discussed, which have not yet been reported to the best of our knowledge.

2. Experimental

2.1. Chemical, reagents, animals and statistical analysis

Reference standards of 20(S)-Rg_3, 20(R)-Rg_3, 20(R)-Rh_2, 20(R)-PPD and 20(S)-PPT (IS) were purchased from Chengdu Must Bio-technology Co., Ltd. (Chengdu, China). Reference standards of 20(S)-Rh_2 and 20(S)-PPD were kindly supplied by Shanghai Pharm Valley Co., Ltd. (Shanghai, China). The purities of 20(S)-Rh_2, 20(R)-Rh_2 and 20(S)-PPD were 98.26%, 92.33% and 95.99%, respectively, and the purities of the other reference standards were all assigned as 98%. Bulk drugs of 20(S)-Rg_3 (purity 96.12%) and 20(R)-Rg_3 (purity 96.16%) for iv and ig administration were obtained by Shanghai Beinuo Biotechnology Co., Ltd. (Shanghai, China). HPLC grade acetonitrile and methanol were obtained from Merck (Darmstadt, Germany). Deionized water was purified using a Milli-Q system (Millipore, Bedford, MA, USA). Other chemicals were all of analytical grade. 24 Sprague-Dawley rats (body weight from 180 to 220 g) were provided by Experimental Center of Nanjing military command (Nanjing, Jiangsu, China), and the studies conformed to the regulations for animal experimentation (State Committee of Science and Technology, China). The pharmacokinetic data were analyzed using WinNolin 6.3 software (Certara, Princeton, USA).

Table 1
MRM paramteters of six compounds and IS (negative ion mode).

Time segment(min)	Compounds	Q1 (*m/z*)	Q3 (*m/z*)	CE (Volts)
4.0	20(S)-Rg_3, 20(R)-Rg_3	783.3	161.0	−35
5.8	20(S)-PPT (IS)	475.4	391.2	−30
8.0	20(S)-Rh_2, 20(R)-Rh_2	681.4	161.0	−23
12.0	20(S)-PPD, 20(R)-PPD	459.3	375.2	−30

2.2. Apparatus and HPLC-MS/MS system

An Agilent 1290 infinity HPLC coupled with Agilent 6490 Triple Quad LC/MS system, and Agilent 1290 infinity LC injector HTC with thermostat (Agilent Technologies, Santa Clara, California, USA) was used. An Inspire C18 column (10cm × 2.1 mm, 3 μm, Dikma, Beijing, China) with a C18 guard column (Security Guard, Phenomenex, USA) was utilized to carry out the separation of Rg_3, Rh_2 and PPD epimers. The temperature of auto-sampler was set at 4 °C. Gradient elution of eluent A: 10 mM ammonium acetate solution (pH value adjusted to 5.0 using acetic acid) and eluent B:acetonitrile was performed. The gradient program was set as follows: 0–2.0 min (40% B), 2.0–5.4 min (40% → 48% B), 5.4–5.5 min (48% → 54% B), 5.5–9.0 min (54% B), 9.0–9.1 min (54% → 75% B), 9.1–15.0 min (75% B). The flow rate was set at 0.6 ml/min and the sample injection volume was 20 μl.

Electrospray ionization (ESI) in negative mode with multiple reaction monitoring (MRM) approach was performed. The parameters were set as follows: gas temperature 290 °C (Nitrogen), gas flow 12 L/min, nebulizer pressure 45 psi, sheath gas temperature 250 °C (Nitrogen), sheath gas flow 11 L/min, capillary voltage −3500 V, nozzle voltage −500 V. The MRM parameters of six analytes and IS are shown in Table 1.

2.3. Drug administration and sample collection

24 Male Sprague-Dawley rats (180–220 g) were assigned randomly into four groups: iv and ig administration of 20(S)-Rg_3 and 20(R)-Rg_3, respectively, with 6 rats for each group. For the dosing solution of iv administration, 50 mg of Rg_3 was firstly dissolved in 2.5 ml of DMSO, and then this solution was dropwisely added to 47.5 ml of 20% Solutol® HS 15 solution prepared by 0.9% NaCl, with votexing all the time. An iv dosage (5 mg/Kg) using about 1 ml of 20(S)-Rg_3 or 20(R)-Rg_3 dosing solution was given to each of 6 rats *via* rail vein within 1 min. For ig administration, Rg_3 was suspended in 0.5% CMC-Na (croscarmellose sodium) aqueous solution by a 30-min votexing to prepare a dosing solution of 5 mg/ml. An ig administration dosage of 50 mg/Kg was given to each rat within 1 min. All the dosing solutions were prepared immediately before administration. 200 μl of blood samples were collected from rat retinal venous plexus at 2, 5, 10, 15, 30 min, 1, 2, 4, 6, 8, 12 and 24 h for iv group, and at 15, 30, 45 min, 2, 4, 6, 8, 12 and 24 h for ig group right after administration. For each blood sample, 10 μl of 0.1% heparin was added and mixed well, and then the blood sample was centrifuged at 1000 × *g* for 5 min to obtain 100 μl plasma.

2.4. Preparation of stock and working solutions

Reference standards of 20(S)-Rg_3, 20(R)-Rg_3, 20(S)-Rh_2, 20(R)-Rh_2, 20(S)-PPD and 20(R)-PPD were accurately weighed into one