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In vivo pharmacokinetic and metabolism studies of ginsenoside Rd

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Abstract

A high-performance liquid chromatography–electrospray ionisation–tandem mass spectrometry (LC–ESI–MS^n) method has been developed to determine ginsenoside Rd in human plasma and to identify its metabolites in rat urine. The plasma and urine samples were pretreated by solid phase extraction (SPE) prior to analyses. In this work, gentiopicroside was used as the internal standard. The lower limit of quantification (LLOQ) for Rd in human plasma was 3 ng/ml. The average half-life time in plasma was detected as 19.29 h, when 10 mg of ginsenoside Rd was administrated intravenously to the volunteers. Seven metabolites including three oxygenated, two combined and two hydrolyzed components were identified in rat urine samples by using LC–MS and MS–MS, when ginsenoside Rd administered either orally or intravenously.

Keywords: In vivo; Pharmacokinetics; Metabolism; Ginsenoside Rd; Liquid chromatography–mass spectrometry

1. Introduction

As a traditional Chinese medicine, Panax ginseng in Asian countries is frequently used to treat many disorders, such as debility, ageing, diabetes, insomnia and sexual inadequacy [1]. The bioactive components of this herbal drug are mainly dammarane triterpene *O*-glycosides, namely, ginsenosides [2–7], of which ginsenoside Rd (Fig. 1) is one of the main active components. Ginsenoside Rd has various bioactivities, in which the corticosterone secretion-inducing activity is very significant [8], hence it has been developed as a novel agent to prevent cardiovascular disease by some manufacturer in China.

Compared with the extensive literatures on phytochemical and pharmacological investigations, there is no investigation reported concerning the pharmacokinetics of ginsenoside Rd, and to our knowledge, only study on its metabolism in vitro by human intestinal bacteria was reported in literature [9]. In vivo metabolism data of ginsenoside Rd may also be valuable for better understanding its pharmacologic activities.

Liquid chromatography coupled to mass spectrometry (LC–MS) is a very powerful and reliable tool for the characterization of chemical constituents [10–13]. Both positive and negative ESI–MS and MS–MS have been successfully applied to analyze various ginsenosides extracted from ginsengs [14–18].

This paper describes a simple, rapid and specific liquid chromatography–mass spectrometry method to determine ginsenoside Rd in human plasma in the positive ion mode, and this method with the lower quantification limit of 3 ng/ml offered satisfactory sensitivity to the pharmacokinetic study of ginsenoside Rd in healthy volunteers. Furthermore, in vivo metabolism of ginsenoside Rd with rat experiments was also first investigated, and seven metabolites of ginsenoside Rd were identified using LC–ESI(-)–MS and MS–MS, thereof three oxygenated, two combined and two hydrolysis metabolites. The oxygenated and hydrolysis metabolites of ginsenoside Rd have never been reported before.

2. Experiment

2.1. Chemicals

20(S)-Ginsenoside Rd (purity 99.3%) was provided by Zhongshan School of Medicine, Sun Yat-sen University (Guangzhou, China). Both 20(S)-ginsenoside Rb_1 (purity

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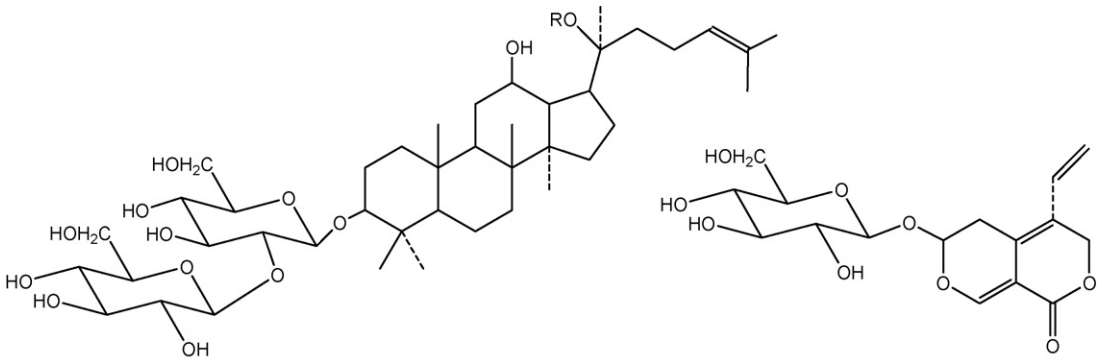

20(S)-ginsenoside Rg3 R=H
20(S)-ginsenoside Rd R=glc-
20(S)-ginsenoside Rb1 R=glc-6"-O-glc-

gentiopicroside

Fig. 1. Structures of 20(S)-ginsenoside Rd, Rb_1, Rg_3 and gentiopicroside (IS).

99.8%) and gentiopicroside (purity 99.8%) as the internal standard (IS) were purchased from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). 20(S)-Ginsenoside Rg_3 (purity 97.0%) was provided by Chromap Institute of Herbal Medicine Research (Zhuhai, China). HPLC-grade methanol was purchased from Fisher Chemicals (Fair lawn, New Jersey, USA). HPLC-grade ammonium acetate was purchased from Tedia Company, Inc. (Fairfield, OH, USA). The water used in the experiments was doubly distilled in the laboratory. Other chemicals (analytical grade) were purchased from Guangzhou Chemical Reagent Factory (Guangzhou, China).

2.2. Chromatographic conditions

HPLC system consisted of Agilent 1100 series HPLC pump and autosamplers (Agilent Technologies, Palo Alto, CA, USA). Ambient temperature was controlled at 18 °C by air conditioner.

In the pharmacokinetic study, the separation was performed on a CAPCELL PAK TYPE UG120 packed column (5 μm, 150 mm × 2.0 mm i.d., Shiseido, Japan), equipped with an ODS guard column (4 mm × 2.0 mm i.d., Phenomenex, Torrance, CA, USA) at 20 °C. An isocratic elution mode was adopted with the mobile phase consisting of 75% methanol, 10% acetonitrile and 15% 10 mM ammonium acetate (v/v/v). The flow rate was 0.25 ml/min.

In the metabolism study, the rat urine samples were chromatographed on an Agilent SB C_{18} column (5 μm, 150 mm × 4.6 mm i.d., Agilent, USA) at 20 °C. The mobile phase consisted of methanol (A) and 10 mM ammonium acetate (B) with a flow rate of 0.8 ml/min. A gradient procedure was as follows: the organic phase A was 50% initially, and then changed to 90% within 40 min and held for 15 min. The LC effluent was introduced into the ESI source in a post-column splitting ratio of 50:1.

2.3. Mass spectrometer conditions

In the pharmacokinetic study, the positive ion mode was used to analyze Rd in human plasma samples. Mass spectrometric experiments were performed on a triple-quadrupole tandem mass spectrometer (API-3000 Pulsar *i*, Applied Biosystems, Foster City, USA). Probe temperature was set at 300 °C with ultrahigh-purity nitrogen as curtain gas (CUR, 12 psi), nebulizer gas (NEB, GAS_1, 6 psi) and Auxiliary gas (nitrogen, AUX, GAS_2, about 7.5 l/min). The ionspray voltage, deflector voltage (DF) and multiplier voltage (CEM) were set at 5000, −200 and 1800 V, respectively, and the declustering potential (DP), focusing potential (FP), entrance potential (EP), collision cell exit potential (CXP) and collision energy (CE) were set at 30, 300, 10, 10 and 20 V in turn. The fragmentation transitions for the multiple reaction monitoring (MRM) were *m/z* 964.6–767.5 for Rd and *m/z* 374.1–195.1 for the IS, with a dwell time of 150 ms per transition.

In the metabolism study, the negative ion mode was used to analyze Rd and its metabolites in all rat urine samples. Mass spectrometric experiments were performed on a quadrupole-time of flight (Q-TOF) tandem mass spectrometer (API Q-STAR Pulsar *i*, Applied Biosystems). The ESI parameters were as follows: ionspray voltage, −4000 V; declustering potential 1 (DP1), −15 V; focusing potential (FP), −80 V and declustering potential 2 (DP2), −15 V. The ion source gas 1 (GAS1), curtain gas (CUR) and collision gas (CAD) were 35, 20 and 5 (arbitrary units), respectively. For full-scan MS analysis, the spectra were recorded in the *m/z* range from 400 to 1200.

2.4. Study on pharmacokinetics of ginsenoside Rd

2.4.1. Plasma calibration curve

The stock solution for 20(S)-ginsenoside Rd was prepared by dissolving the accurately weighed reference compound in methanol at a concentration of 1 mg/ml and stored at 4 °C. The solution was then diluted with methanol into the standard working solutions at concentrations of 0.3, 0.5, 1, 3, 10, 30, 100, 400 and 500 μg/ml. A 200 ng/ml working solution for internal standard was prepared by diluting the 20 μg/ml stock solution of gentiopicroside with methanol.

Plasma standard samples were prepared using drug-free human plasma at concentrations of 3, 5, 10, 30, 100, 300,

1000, 4000 and 5000 ng/ml for ginsenoside Rd. Quality control samples were prepared at concentrations of 8, 800, 2000 and 4000 ng/ml in blank human plasma. A 0.3 ml of thawed plasma sample was transferred to a plastic test tube as well as 0.3 ml water and 0.1 ml internal standard solution. After vortex-mixed for 30 s, an aliquot of 650 μl of the sample was loaded on HLB cartridge (1cc, Oasis^{TM}, Waters, USA) preconditioned with 1 ml of methanol followed by 1.5 ml of water. The cartridge loaded with sample was washed with 1 ml of water. Vacuum was then applied for 3 min to remove the aqueous part. Rd and IS were eluted with 1 ml of methanol, and 0.3 ml of the eluate was transferred into an eppendorff tube, and then to the same tube 100 μl of solution consisting of 10 mM ammonium acetate and acetonitrile (v/v 3:2) was added. The samples were well mixed and centrifuged at 10,800 × *g* for 3 min. A 5 μl-aliquot of the supernatant was analyzed using LC–MS.

2.4.2. Study on pharmacokinetics of ginsenoside Rd in healthy volunteers

Ten Chinese healthy volunteers (five females and five males) were involved in this study. The mean age was 25 ± 5 years and the body weight was over 45 kg with a body mass index range of 19–25 kg/m^2. The exclusion criteria included history or evidence of cardiovascular, hepatic, renal, gastrointestinal or hematologic deviations or any acute or chronic diseases or drug allergy. All volunteers were instructed to abstain from taking any medication from 2 weeks before and during the study. A single dose of Rd (10 mg) injection was diluted into 50 ml of solution with nature saline containing 10% 1, 3-propanediol (v/v). After overnight fasted, each volunteer was dosed with the solution via intravenously guttae for 30 min. Water intake was allowed 2 h after dosing and low-fat standard meals were provided to volunteers 4 and 10 h post-dose. The volunteers were prohibited from smoking, alcohol, caffeine and strenuous activity during the study and were under direct medical supervision at the study site. Venous blood (5 ml) was withdrawn from every volunteer pre-dose, every 10 min through to 0.5 h post-dose and 1, 1.5, 2.5, 4.5, 6.5, 8.5, 12.5, 16.5, 24.5, 36.5, 48.5, 60.5, 72.5, 84.5 and 96.5 h post-dose, and transferred immediately to the heparinized tubes. After spiking, the blood samples were kept at room temperature for 30 min, and then centrifuged for 15 min. Plasma was separated and stored at −80 °C until use. Plasma samples were prepared for the LC–MS analysis by using the same procedure described above.

2.5. Urine sample preparation in the metabolism study of ginsenoside Rd

In this study, six male Sprague–Dawley rats (body weight 200–220 g) were halved into two groups at random and were intravenously and orally administrated with ginsenoside Rd, respectively. The reference standard Rd (600 mg) was dissolved in 10 ml of nature saline containing 20% 1,3-propanediol (v/v). Each of the intravenous group was dosed with 1 ml of this solution within 1 min. Oral administration of Rd (150 mg/kg) was conducted on the other group. Rat urine samples were collected from 0 to 24 h after administration. Each urine sample was concentrated under reduced pressure, and then the concentrated sample was added onto the HLB cartridge (1cc, Oasis^{TM}, Waters) that had been conditioned with 1 ml methanol and 1.5 ml water in turn. After the sample was drawn through the cartridge, the cartridge was washed with 1 ml of water. Vacuum was then applied for 3 min to remove the aqueous part. The analytes were then eluted with 1.5 ml methanol. The eluate was transferred to a clean test tube and evaporated to dryness under a gentle stream of nitrogen at 35 °C. The residue was reconstituted in 300 μl of 50% methanol (v/v) and centrifuged by a micro-spin filter tube (0.22 μm nylon, Alltech) at 10,800 × *g* for 1 min.

3. Results and discussion

3.1. LC–MS analysis of ginsenoside Rd in human plasma samples

The specificity of the method was demonstrated by comparing MRM chromatograms for reference standard (Rd and the IS) for a drug-free plasma sample, a spiked plasma sample and a plasma sample from a healthy volunteer 6.5 h after intravenous administration. As shown in Fig. 2, no significant peaks interfering with analytes were observed in the drug-free human plasma.

Linear calibration curves with correlation coefficients greater than 0.9977 were obtained in the concentration ranges 3–5000 ng/ml in plasma for Rd. The lower limit of quantification (LLOQ), defined as the lowest concentration analyzed with accuracy within ±15% and a precision ≤15%, was 3 ng/ml for determination of ginsenoside Rd in plasma.

Precision and accuracy of the assay were determined by replicate analyses ($n = 5$) of QC samples at four concentrations, by performing the complete analytical runs on the same day and also on 5 consecutive days. The data from these QC samples were examined by a one-way analysis of variance (ANOVA). The intra-day and inter-day precisions were less than 10% for each QC level of Rd. The accuracy, determined from QC samples, was within ±3% for each QC level.

The extraction recoveries of ginsenoside Rd were determined by comparing the peak area of Rd in plasma samples that had been spiked with the analyte ante-extraction with that for samples to which Rd had been added post-extraction. As a result, the extraction recoveries of Rd were 83.39, 79.36, 81.01 and 83.07% at concentrations of 8, 800, 2000 and 4000 ng/ml.

The stability of Rd in human plasma was investigated under a variety of storage and process conditions. Rd in human plasma was found to be stable after three cycles of freeze–thaw and for at least 72 h at room temperature (25 °C). No significant change was found in reconstituted reagent after sample had been prepared for 8 h at room temperature and results of long-term stability showed that Rd was still stable for at least 5 months at −80 °C. This indicated that under the current experimental conditions, Rd as the analytical index was stable.

The plasma samples in the pharmacokinetic study were analyzed by the present LC–MS–MS method. Fig. 3 showed the mean plasma concentration–time curve of Rd ($n = 10$); the pharmacokinetic parameters are presented in Table 1. After the

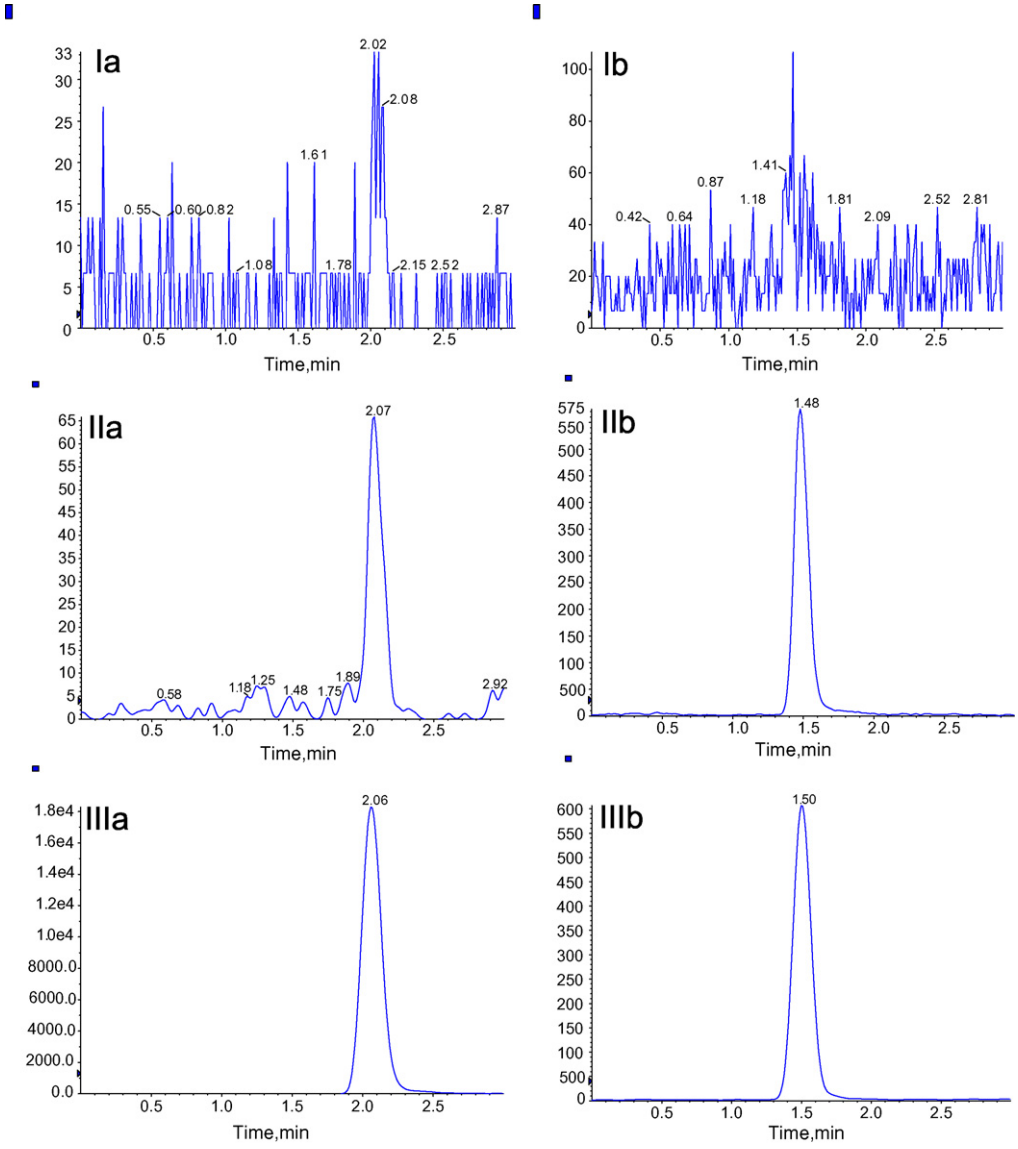

Fig. 2. MRM chromatograms of Rd and IS (a) Rd (*m/z* 964.6/767.5); (b) IS (*m/z* 374.1/195.1): (I) drug-free plasma; (II) plasma spiked with 3 ng/ml of Rd and 200 ng/ml of IS and (III) a plasma sample (1079.95 ng/ml) 6.5 h after intravenous administration.

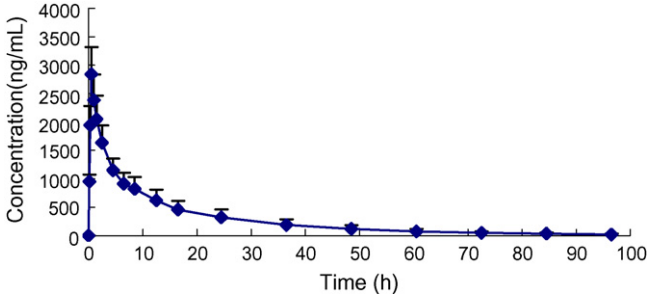

Fig. 3. Mean plasma concentration–time profile of ginsenoside Rd in human after a single dose of 10 mg ($n = 10$).

Table 1
The main pharmocokinetic parameters of ginsenoside Rd after i.v. administrations of a single dose of 10 mg to 10 healthy volunteers (mean ± S.D., $n = 10$)

Parameters	Ginsenoside Rd
C_{max} (ng/ml)	2841.18 ± 473.03
T_{max} (h)	0.50 ± 0.00
$t_{1/2}$ (h)	19.29 ± 3.44
AUC_{0-t} (ng h/ml)	27261.63 ± 8116.88
$AUC_{0-\infty}$ (ng h/ml)	27929.39 ± 8615.75
MRT_{0-t} (h)	17.52 ± 3.73
$MRT_{0-\infty}$ (h)	19.79 ± 5.24
V_d (l/kg)	10.57 ± 2.88
CL (l/h)	0.39 ± 0.12

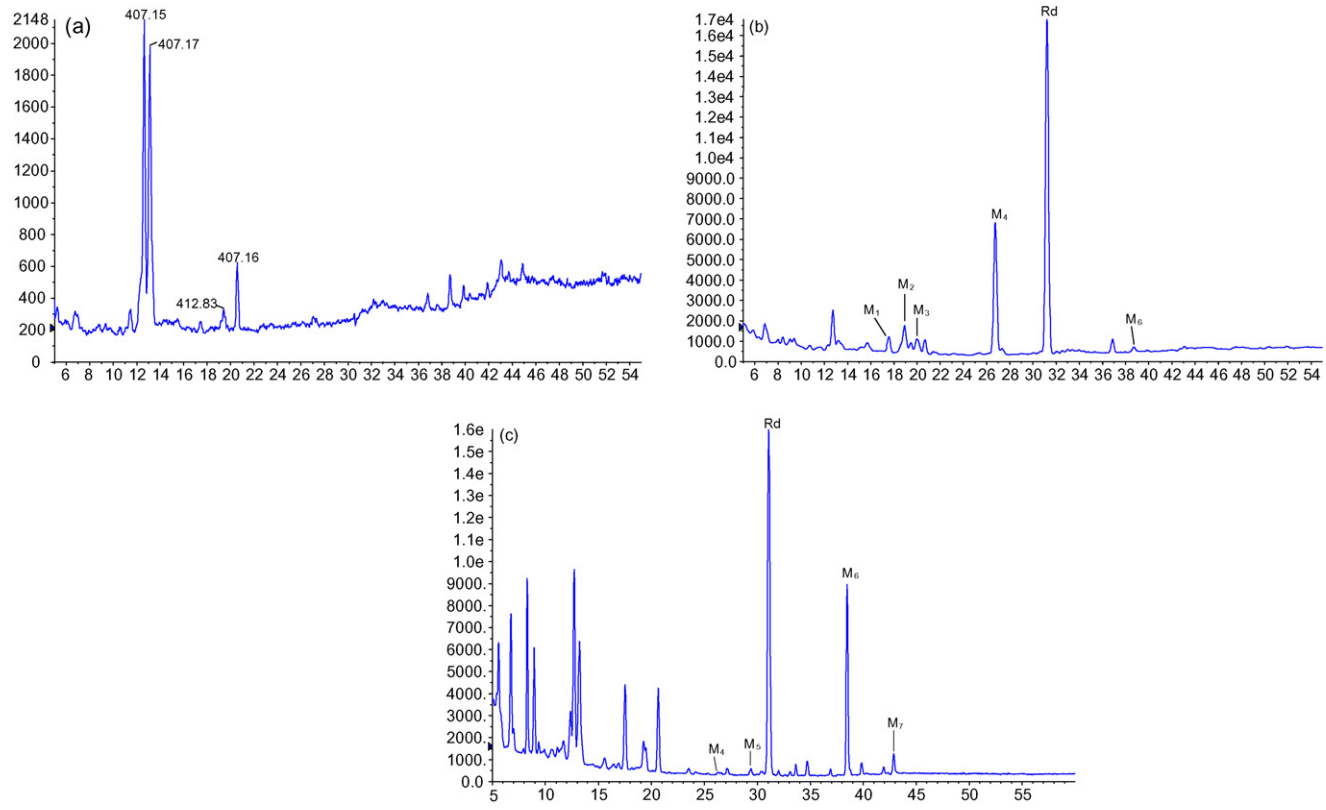

Fig. 4. Total ion chromatogram of rat urine: (a) blank urine sample; (b) urine sample after intravenous administration and (c) urine sample after oral administration.

intravenous dose of 10 mg, the mean maximum plasma concentration (standard deviation, S.D.) of Rd was 2841.18 ng/ml (±473.03) occurring at 0.5 h post-dose. The mean apparent plasma half-life (S.D.) was 19.29 h (±3.44). The mean (S.D.) area under the plasma concentration versus time curve was 27261.63 ng h/ml (±8116.88).

3.2. Metabolism study of ginsenoside Rd in rat urine samples

Seven possible metabolites of ginsenoside Rd were detected in rat urine samples collected from 0 to 24 h after intravenous and oral administrations of this compound. The metabolites were detected by the LC–MS analyses and further confirmed with the MS–MS experiments. Oxygenation, deglycosylation and combination were found to be the major metabolic pathways of Rd.

Fig. 4 showed the total base ion mass chromatograms of rat urine samples. Several oxygenated metabolites of Rd were detected, thereof three major peaks (M_1, M_2 and M_3, t_R = 17.32, 18.38 and 19.45 min, respectively) were identified as monooxygenated metabolites of Rd by the negative ion ESI–MS–MS analyses, whereas other minor peaks could not be confirmed because of no good MS–MS spectra obtained from the ion peaks with very low intensity. All the M_1, M_2 and M_3 exhibit $[M-H]^-$ ions at *m/z* 961, whose molecular masses are 16 Da greater than that of ginsenoside Rd. The MS–MS spectra of three deprotonated metabolites and Rd were shown as Fig. 5. The MS–MS spectrum of the $[M-H]^-$ ion of Rd showed three major product ions at *m/z* 783, 621 and 459, resulting from the neutral loss of one, two and three glucose residues, respectively. Similarly, the MS–MS spectra of the deprotonated molecules of M_1, M_2 and M_3 also yielded the deglycosylated product ions at *m/z* 799, 637 and 475. The fragment ion at *m/z* 161 was observed in their MS–MS spectra, corresponding to the glucose moiety of these compounds.

Although the exact oxygenation site of the metabolites M_1, M_2 and M_3 could not be determined, their MS–MS data provided evidence of possible oxygenation on the top-right aliphatic chain. The loss of $-CH_2CH_2CH{=}C(CH_3)_2$ moiety (*m/z* 621 → 537 and *m/z* 459 → 375) was observed in the MS–MS spectrum of ginsenoside Rd, while the MS–MS spectra of M_1, M_2 and M_3 showed the loss of 100 Da (*m/z* 637 → 537 and *m/z* 475 → 375) but no loss of 84 Da, suggesting the neutral loss of the monooxygenated aliphatic chain.

The metabolite M_4 was identified as ginsenoside Rb_1 in comparison with the reference standard. As an isomer of Rb_1, the molecular mass of M_5 is also 1108 Da. Both of them are the Phase II combined metabolites of Rd, i.e. they were the glycosylated products of Rd. Like ginsenoside Rd, the characteristic product ions at *m/z* 945, 783, 621 and 459 also appeared in the MS–MS spectrum of their deprotonated molecules (Fig. 5E and F).

The $[M-H]^-$ ion of M_6 was at *m/z* 783 (162 Da less than that of Rd). It was identified as ginsenoside Rg_3 by comparing with the authentic standard. The ESI–MS–MS spectrum in Fig. 5G displayed that major product ions at *m/z* 621 and

459 most likely resulted from the loss of one and two sugars from the deprotonated molecular ion. The characteristic product ion at *m/z* 375 was inferred to be produced by the loss of the $-CH_2CH_2CH{=}C(CH_3)_2$ moiety (84 Da) from the *m/z* 459 ion. The deprotonated ion peaks of glucose moiety at *m/z* 161 and 323 were also observed in the MS–MS spectrum.

As one of the major metabolites of Rd, the molecular mass of M_7 was 324 Da less than that of Rd, consistent with two glucose moieties. The product ions at *m/z* 459 most likely originated from the neutral loss of a hexose residue (162 Da). Moreover, the product ion at *m/z* 375 corresponded to the farther loss of the aliphatic side chain (*m/z* 459 → 375). Based on considerations

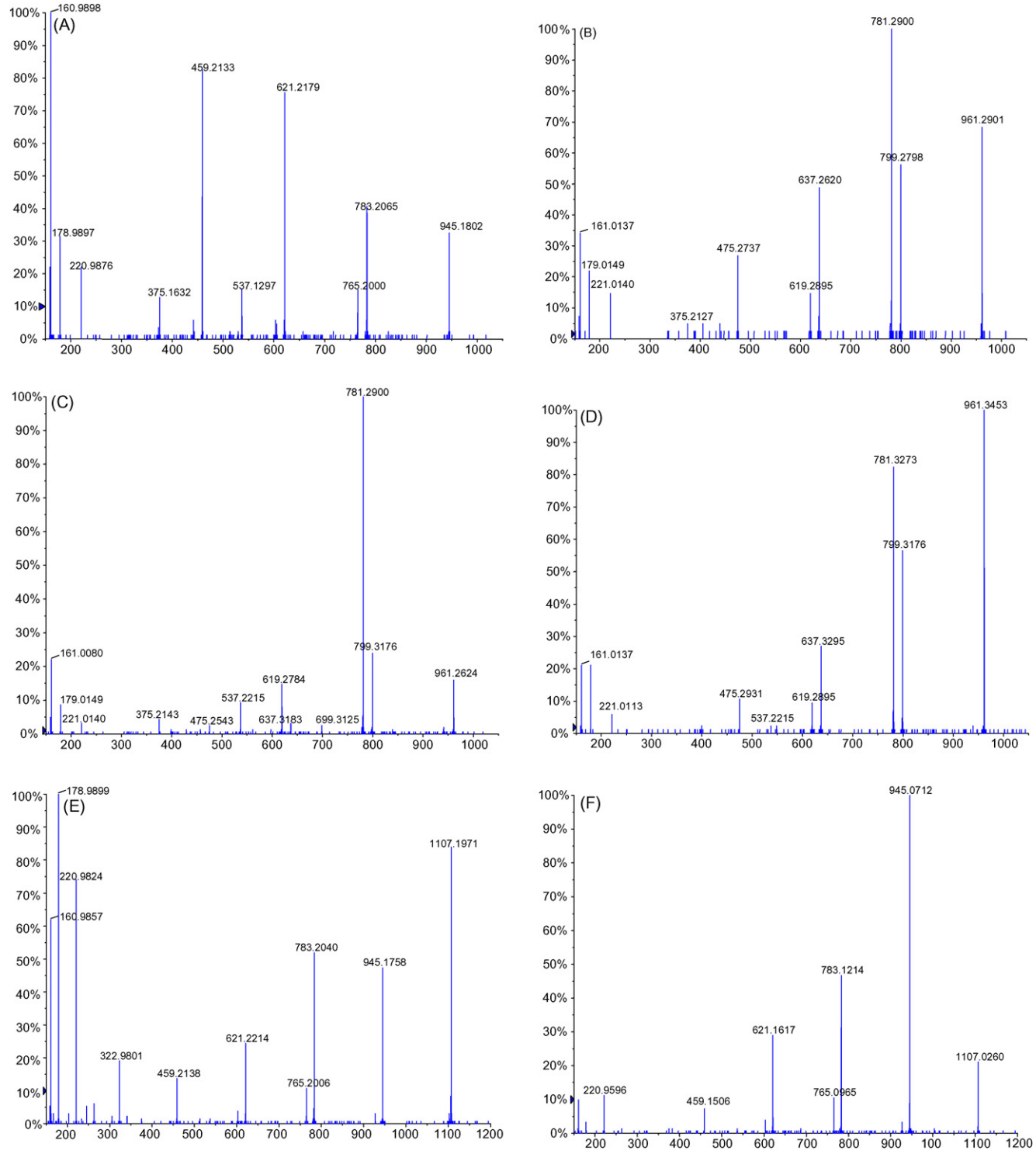

Fig. 5. Negative ion ESI–MS–MS spectra of ginsenoside Rd and its metabolites: (A) MS–MS spectrum of ginsenoside Rd; (B) MS–MS spectrum of M_1 (monooxygenated Rd); (C) MS–MS spectrum of M_2 (monooxygenated Rd); (D) MS–MS spectrum of M_3 (monooxygenated Rd); (E) MS–MS spectrum of M_4 (ginsenoside Rb_1); (F) MS–MS spectrum of M_5 (iso-ginsenosideRb_1); (G) MS–MS spectrum of M_6 (ginsenoside Rg_3) and (H) MS–MS spectrum of M_7 (ginsenoside Rh_2).

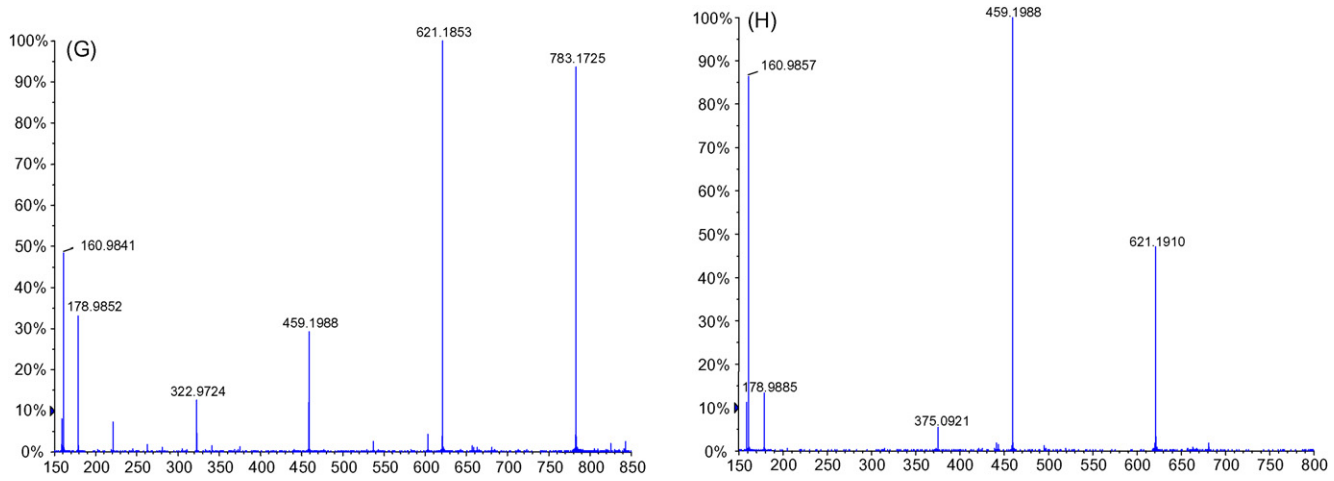

Fig. 5. (*Continued*).

of biosynthesis, i.e. those structures that had been reported previously in phytochemical and metabolic investigation of ginseng, M_7 was preferentially identified as Rh_2.

Fig. 6 summarizes the major metabolites of Rd as detected in rat urine samples and its metabolic pathway. The total ion chromatograms showed that most amount of ginsneoside-Rd remained unchanged and excreted through rat urine, only a little amount—Rd was subjected to change during metabolic process in human (Fig. 4). It is also worth notice in Fig. 6 that the metabolic pathways of ginsenoside Rd looked different between the different administration routes, among the metabolic compounds, M_4 dominated the profile obtained from intravenous administration, and M_6 dominated the profile from oral administration (Fig. 4). That hinted that the glycosylation process of ginsenoside Rd only occurred through intravenous administration but the hydrolyzed metabolic process mainly happened through stomach-intestine-liver-system via oral administration.

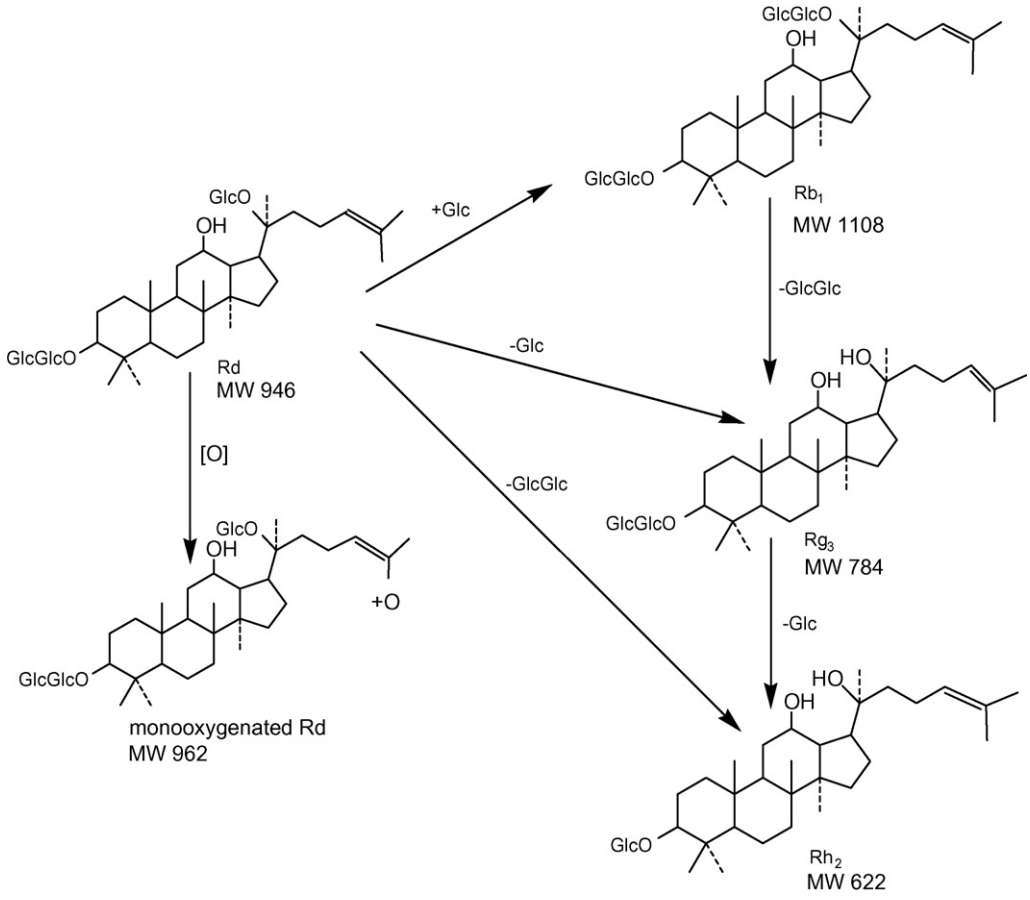

Fig. 6. Proposed major metabolic pathway of ginsenoside Rd in rat.

4. Conclusion

In conclusion, the present optimized method was validated to guarantee a reliable determination of ginsenoside Rd in human plasma. It was then successfully applied to a pharmacokinetic study of Rd after intravenous administrations. The long half-life of Rd from the human PK study indicated that the ginsenoside Rd may be metabolized slowly after intravenous administration. Seven metabolites of Rd were detected from rat urine collected from 0 to 24 h after oral and intravenous administration. The metabolites detected by LC–MS were confirmed by the MS–MS analyses and reference compounds. Oxygenation, glycosylation were found to be the major metabolic pathway of Rd in rats administrated intravenously, while deglycosylation of Rd was the major metabolic mode in rats after oral dosing. The identification of metabolites and the presumed metabolic pathways may provide important information to understand the bioactive form of the ginsenoside.

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