

Article

## Metabolism Study of Notoginsenoside R<sub>1</sub>, Ginsenoside Rg<sub>1</sub> and Ginsenoside Rb<sub>1</sub> of Radix Panax Notoginseng in Zebrafish

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**Abstract:** Zebrafish, a common model organism for studies of vertebrate development and gene function, has been used in pharmaceutical research as a new and powerful tool in recent years. In the present study, we applied zebrafish for the first time in a metabolic study of notoginsenoside (R<sub>1</sub>), ginsenoside (Rg<sub>1</sub>) and ginsenoside (Rb<sub>1</sub>), which are saponins isolated from Panax notoginseng. Metabolites of these three saponin compounds in zebrafish after exposure for 24 h were identified by high performance liquid chromatography - electrospray mass spectrometry (HPLC-ESI-MS) with a Zorbax C-18 column for separation using a binary gradient elution of 0.05% formic acid acetonitrile - 0.05% formic acid water. The quasi-molecular ions of compounds were detected in negative mode. Step-wise deglycosylation metabolites and hydroxylation metabolites of the three saponins were found, which were coincide with regular methods for metabolic analysis. Our study demonstrated that the zebrafish model can successfully imitate the current metabolic model with advantages of lower cost, far less amount of compound

needed, easy set up and high performance. Our data suggests that the zebrafish metabolic model has the potential for developing a novel method for quickly predicting the metabolism of Chinese herb components, including those of trace compounds.

**Keywords:** zebrafish; notoginsenoside R<sub>1</sub>; ginsenoside Rg<sub>1</sub>; ginsenoside Rb<sub>1</sub>; metabolism

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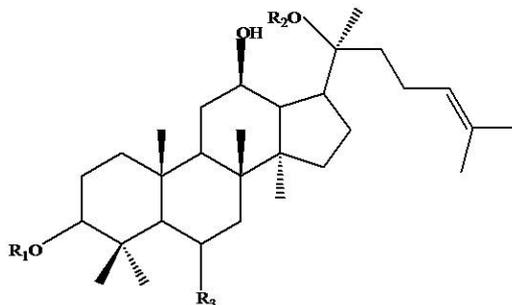
## 1. Introduction

In recent years, metabolic studies of Traditional Chinese Medicine (TCMs) have been applied effectively in the major fields of TCM research, including material basis, mechanism of action and quality control. However, there are still limitations for metabolic study methods so far using either *in vivo* or *in vitro* models in TCM research: the *in vivo* method is resource costly, involving consumption of a great amount of compound which is difficult to purify from Chinese herbs, and many trace components cannot even be evaluated. On the other hand, the *in vitro* method is expensive due to the high standard of experimental conditions, which makes it hard to perform in some laboratories. Therefore, it is really important to establish an alternative metabolic study method for TCMs, which would combine advantages of these methods and overcome compound shortages to make it available for even trace compounds analysis.

Over the past ten years, zebrafish has been used as a popular model organism in diverse fields of research such as developmental and evolutionary biology, toxicology and pharmacology. They are considered a new powerful tool, especially in pharmaceutical research [1-7], because they are genetically similar to humans and have the same complex organs found in mammals. Besides, it is known there are intestinal bacteria in zebrafish [8] and P450s enzymes are expressed [9,10], which make them suitable for study of drug metabolism. Based on above advantages of zebrafish, we put forward the ideas for metabolic study of TCMs using zebrafish for the first time [11,12].

Panax notoginseng, a well known Chinese medicinal herb named Sanqi, has been widely used for the treatment of analgesia, hemostasis, cardiovascular and cerebrovascular diseases. Notoginsenoside R<sub>1</sub> (R<sub>1</sub>), ginsenoside Rg<sub>1</sub> (Rg<sub>1</sub>) and ginsenoside Rb<sub>1</sub> (Rb<sub>1</sub>) (Figure 1) are the major bioactive saponins of Sanqi monitored for quality control [13]. A number of metabolic studies of these three saponins *in vivo* or *in vitro* have been reported together with their metabolic mechanisms, which were demonstrated to involve stepwise deglycosylation and hydroxylation. For example, metabolites of R<sub>1</sub> are ginsenoside Rg<sub>1</sub>, notoginsenoside R<sub>2</sub>, ginsenoside Rh<sub>1</sub> or F<sub>1</sub>, protopanaxatriol (ppt) and hydroxynotoginsenoside R<sub>1</sub> [14-16]; metabolites of Rg<sub>1</sub> are ginsenoside Rh<sub>1</sub> or F<sub>1</sub>, protopanaxatriol (ppt) [17-22]; and metabolites of Rb<sub>1</sub> are ginsenoside Rd, ginsenoside Rg<sub>3</sub> or F<sub>2</sub>, ginsenoside Rh<sub>2</sub>, hydroxyginsenoside Rb<sub>1</sub> and protopanaxadiol (ppd) [23-28]. For the first time, we have investigated a new method using zebrafish as a model in the metabolic study of TCM components due to the advantages of zebrafish. In the present study, notoginsenoside R<sub>1</sub>, ginsenoside Rg<sub>1</sub> and ginsenoside Rb<sub>1</sub> whose metabolic mechanisms have been clearly elucidated were selected as sample compounds, and high performance liquid chromatography—electrospray mass spectrometry (HPLC-ESI-MS) was used for analysis of their metabolites after zebrafish exposure.

**Figure 1.** Structures of notoginsenoside R<sub>1</sub>, ginsenoside Rg<sub>1</sub> and ginsenoside Rb<sub>1</sub> in Radix Panax notoginseng in this study.



notoginsenoside R<sub>1</sub> (R<sub>1</sub>): R<sub>1</sub> = H, R<sub>2</sub> = Glc, R<sub>3</sub> = O-Glc<sup>2</sup>-Xyl;

ginsenoside Rg<sub>1</sub> (Rg<sub>1</sub>): R<sub>1</sub> = H, R<sub>2</sub> = Glc, R<sub>3</sub> = O-Glc;

ginsenoside Rb<sub>1</sub> (Rb<sub>1</sub>): R<sub>1</sub> = -Glc<sup>2</sup>-Glc, R<sub>2</sub> = -Glc<sup>6</sup>-Glc, R<sub>3</sub> = H;

abbreviations: Glc, β-D-glucose; Xyl, β-D-xylopyranosyl.

Based on results from zebrafish metabolic study model and existing *in vivo* and *in vitro* study methods, we report evidence for a feasible and economic model, and our research should contribute to developing a novel, simple, low cost, high-performance metabolic study model for trace components of TCMs.

## 2. Results and Discussion

### 2.1. Conditions Selected for Zebrafish Metabolism Experiments

Method for drug administration and sampling: due to the small size of zebrafish, it is difficult to administer small quantities of drugs and to obtain blood samples for trace component analysis. But if zebrafish are exposed to drug solutions, they can absorb compounds from solution, and the metabolites transformed by zebrafish will be continuously output into the solution, so analysis of component changes in solution and whole zebrafish bodies can provide some information about drug metabolism, and this method is simple and feasible.

Solution concentration of compounds should not influence zebrafish activity for at least 24 h; the water temperature should be controlled within a suitable living temperature for zebrafish (from 20 °C to 26 °C; the present experiments were performed in a 23 °C thermostated waterbath); taking factors such as the stability of blank drug solution and zebrafish activity into consideration, and referring to commonly sampling within 24 h of mammalian metabolism experiments, we selected 24 h as sampling time after zebrafish exposure to compounds, thus the accumulation of metabolites may satisfy the demand for detection, and usage amount of compounds would be minimized. The R<sub>1</sub>, Rg<sub>1</sub> and Rb<sub>1</sub> required in the present test was only 9.63, 16.75 and 21.24 μg/mL, respectively.

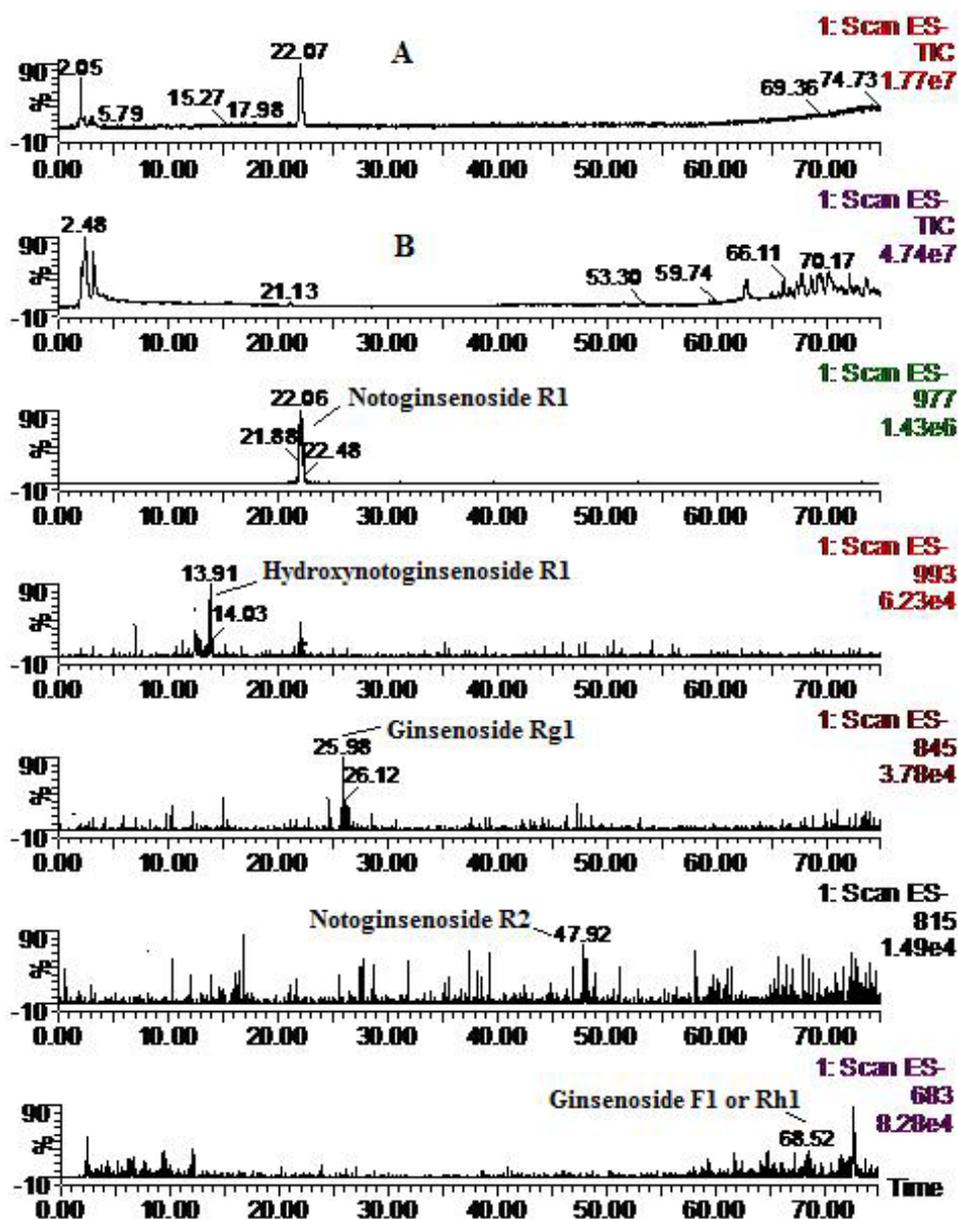
### 2.2. Analysis of Metabolic Components of Notoginsenoside R<sub>1</sub>, Ginsenoside Rg<sub>1</sub> and Ginsenoside Rb<sub>1</sub> after Zebrafish Exposure by HPLC-ESI-MS

R<sub>1</sub>, Rg<sub>1</sub>, Rb<sub>1</sub> and their metabolites after zebrafish exposure were identified by HPLC-ESI-MS with an ESI source in negative mode. Consistent with reference [29] the saponins and their metabolites

exhibited their quasi-molecular ions of  $[M-H]^-$  and  $[M+HCOO]^-$  for molecule mass information. By extracting the ion current, attentive study of the mass spectra of compounds and comparison with reference data and some standards, both stepwise deglycosylation and hydroxylation metabolites of  $R_1$ ,  $R_{g1}$  and  $R_{b1}$  were identified by comparing with blank samples.

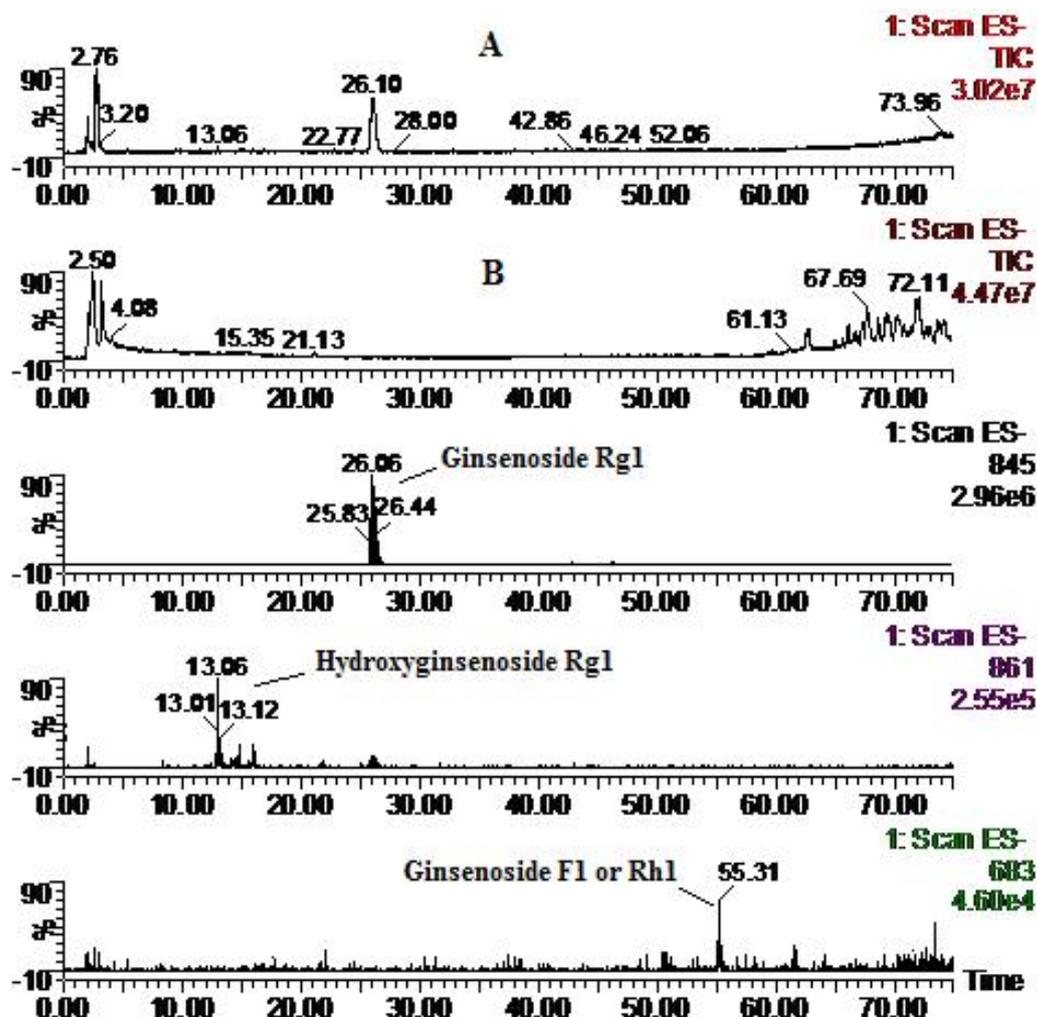
Four metabolites of  $R_1$  were identified, in addition to the parent component notoginsenoside  $R_1$  (MW 932) (Figure 2), including hydroxynotoginsenoside  $R_1$  (MW 948), notoginsenoside  $R_2$  (MW 770) degradation products derived from  $R_1$  via cleavage of one molecule of glucose, ginsenoside  $R_{g1}$  (MW 800) derived from  $R_1$  via cleavage of one xylose moiety, and ginsenoside  $F_1$  or  $R_{h1}$  (MW 638), monoglucosylated protopanaxatriol ginsenoside, derived from  $R_1$  via cleavage of both glucose and xylose moieties. These results were consistent with previous reports on mammalian metabolism [14-16].

**Figure 2.** Total ion chromatogram (A: solution; and B: zebrafish) and extracted ion chromatograms for notoginsenoside  $R_1$  after zebrafish exposure for 24 h.



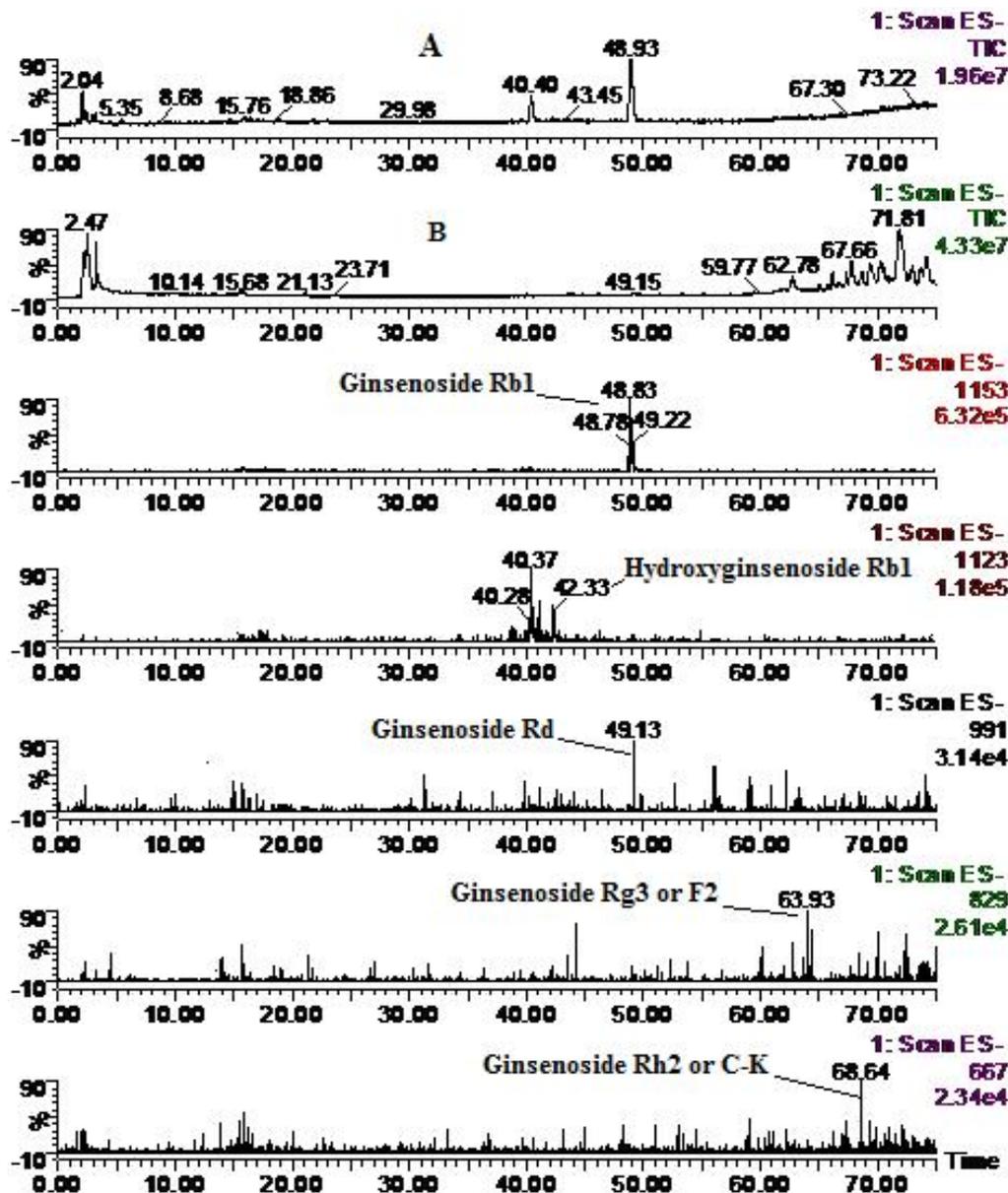
Two metabolites of Rg<sub>1</sub> were identified, in addition to the parent component ginsenoside Rg<sub>1</sub> (MW 800) (Figure 3), including hydroxyginsenoside Rg<sub>1</sub> (MW 816), and a monoglucosylated protopanaxatriol ginsenoside named ginsenoside F<sub>1</sub> or Rh<sub>1</sub> (MW 638) derived from Rg<sub>1</sub> *via* cleavage of a glucose moiety. These results are consistent with previous reports concerning the rat metabolism [17-21,29].

**Figure 3.** Total ion chromatogram (A: solution; and B: zebrafish) and extracted ion chromatograms for ginsenoside Rg<sub>1</sub> after zebrafish exposure for 24 h.



Four metabolites of Rb<sub>1</sub> were identified in addition to the parent component ginsenoside Rb<sub>1</sub> (MW 1108) (Figure 4), including hydroxyginsenoside Rb<sub>1</sub> (MW 1124), ginsenoside Rd (MW 946) obtained from Rb<sub>1</sub> *via* cleavage of one molecule of glucose, ginsenoside Rg<sub>3</sub> or F<sub>2</sub> (MW 784) derived from Rb<sub>1</sub> *via* cleavage of two glucose moieties, and ginsenoside Rh<sub>2</sub> or C-K (MW 622), a monoglucosylated protopanaxadiol ginsenoside, derived from Rb<sub>1</sub> *via* cleavage of three glucose moieties. These results were consistent with previous reports on rat metabolism [25,28].

**Figure 4.** Total ion chromatogram (A: solution; and B: zebrafish) and extracted ion chromatograms for ginsenoside Rb<sub>1</sub> after zebrafish exposure for 24 h.



MS data for R<sub>1</sub>, Rg<sub>1</sub>, Rb<sub>1</sub> and their metabolites by zebrafish are shown in Table 1, the representative MS spectra are shown in Figure 5, and the possible metabolic pathways of R<sub>1</sub>, Rg<sub>1</sub> and Rb<sub>1</sub> are elucidated in Figure 6.

**Table 1.** MS data for R<sub>1</sub>, Rg<sub>1</sub> and Rb<sub>1</sub> and their metabolites after zebrafish exposure for 24 h.

Compounds	Retention time (min)	quasi-molecular ions peak		MW	Metabolite presumed	Zebrafish		Mammalian metabolism (references)
		[M-H] <sup>-</sup>	[M+HCOO] <sup>-</sup>			solution	body	
R <sub>1</sub>	21.91	931.84	977.73	932.8	Notoginsenoside R <sub>1</sub>	+	+	[14-16]
	13.91	947.71	993.46	948.7	Hydroxynotoginsenoside R <sub>1</sub>	+		[14]
	25.98	799.81	845.83	800.8	Ginsenoside Rg <sub>1</sub>	+		[14, 16]

Table 1. Cont.

	48.07	769.24	815.54	770.2	Notoginsenoside R <sub>2</sub>	+		[15]
	68.52	637.21	683.1	638.2	Ginsenoside F <sub>1</sub> or Rh <sub>1</sub>		+	[14-16]
Rg <sub>1</sub>	26.10	799.81	845.83	800.8	Ginsenoside Rg <sub>1</sub>	+	+	[15-19]
	13.06		861.77	816.8	Hydroxyginsenoside Rg <sub>1</sub>	+		[29]
	55.31	637.69	683.23	638.7	Ginsenoside F <sub>1</sub> or Rh <sub>1</sub>	+		[17-21]
Rb <sub>1</sub>	48.93	1107.78	1153.46	1108.9	Ginsenoside Rb <sub>1</sub>	+	+	[25, 28]
	42.16	1123.03	1169.47	1124.0	Hydroxyginsenoside Rb <sub>1</sub>	+		[28]
	49.13	944.86	991.26	946.0	Ginsenoside Rd	+	+	[25, 28]
	63.98	783.87	829.98	784.8	Ginsenoside Rg <sub>3</sub> or F <sub>2</sub>	+		[25, 28]
	68.66	620.79	666.75	622	Ginsenoside Rh <sub>2</sub> or C-K	+	+	[25, 28]

+ detected.

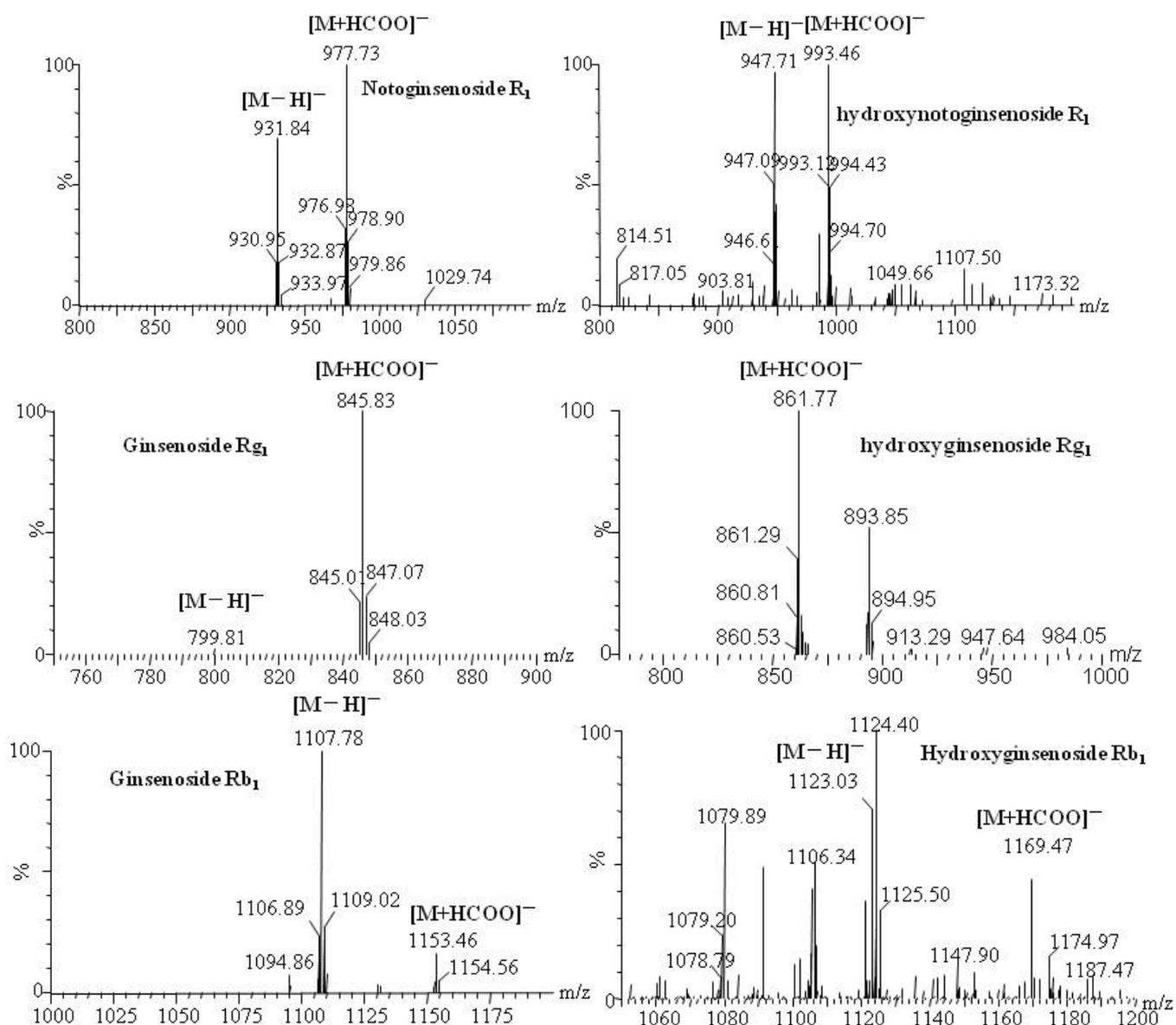
Figure 5. Representative MS spectra of notoginsenoside R<sub>1</sub>, ginsenoside Rg<sub>1</sub> and ginsenoside Rb<sub>1</sub> and their transformative components by zebrafish.

Figure 5. Cont.

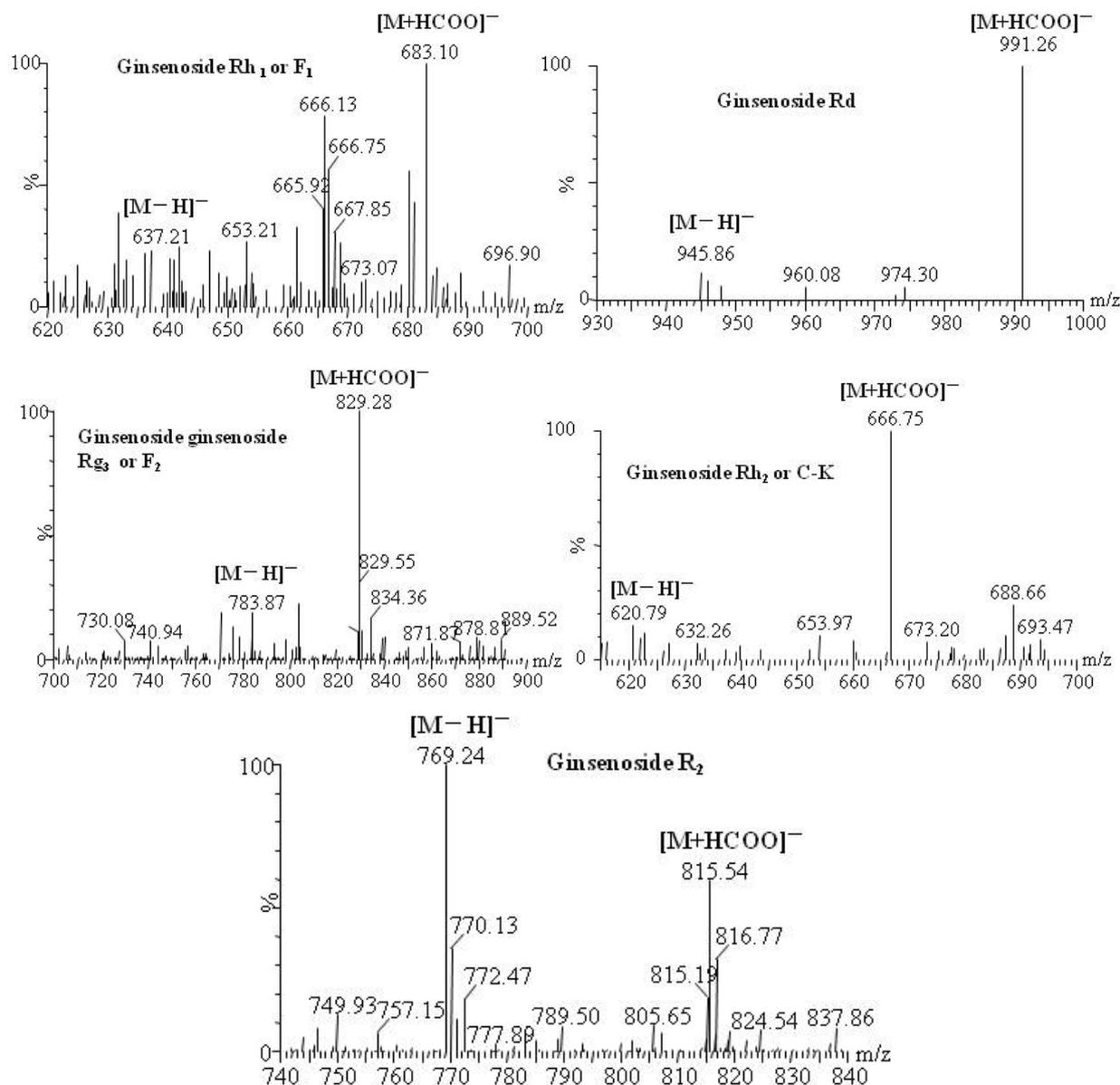
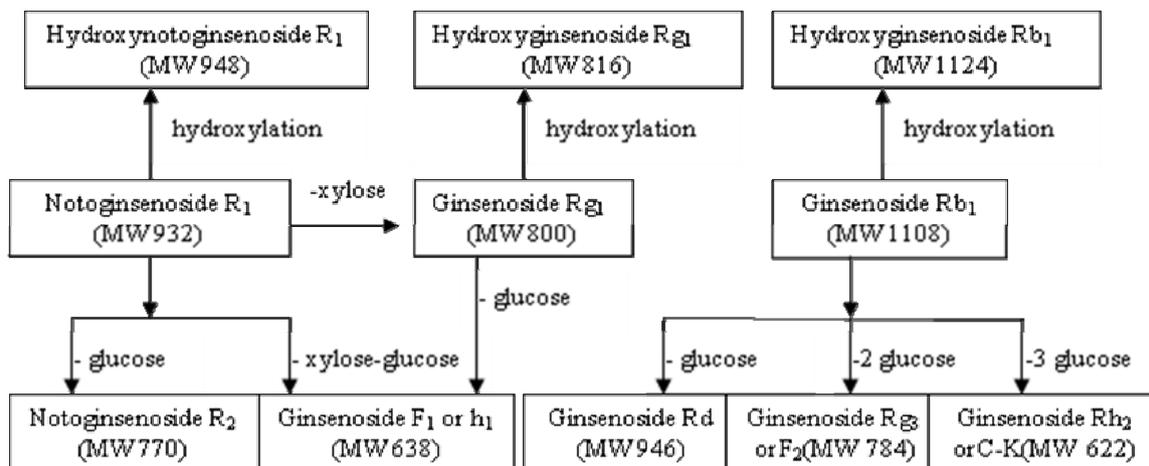


Figure 6. The possible metabolic pathways of notoginsenoside R<sub>1</sub>, ginsenoside R<sub>g1</sub> and ginsenoside R<sub>b1</sub> by zebrafish.



### 2.3. Rationality and Advantages of Metabolic Study with Zebrafish Compared to Existing Model

The metabolic mechanisms of R<sub>1</sub>, Rg<sub>1</sub> and Rb<sub>1</sub> by zebrafish are highly consistent with the existing mammal results; over the past thirty years, a number of *in vivo* or *in vitro* metabolic studies of R<sub>1</sub>, Rg<sub>1</sub> and Rb<sub>1</sub> have been reported, though results of different reports may not consistent with each other totally, the metabolites are similar or complementary, and their metabolic mechanisms can be elucidated as deglycosylation and hydroxylation, among which deglycosylation is the major metabolic pathway of intestinal bacteria, and secondary glycoside metabolites *via* cleavage of one or multiple sugar moieties were reported; hydroxylation is another metabolic pathway, such as seen in the metabolites of hydroxynotoginsenoside R<sub>1</sub> [15] and hydroxyginsenoside Rb<sub>1</sub> [28]. A metabolic study of Fufang Danshen prescription presumed hydroxylation of ginsenoside Rg<sub>1</sub> [29]. Our present study found deglycosylation and hydroxylation of R<sub>1</sub>, Rg<sub>1</sub> and Rb<sub>1</sub> by zebrafish for the first time, which is highly consistent with the results of existing methods: all stepwise deglycosylation metabolites were found, except protopanaxatriol (ppt) and protopanaxadiol (ppd), the reasons maybe that stable monoglucosylated ginsenoside is difficult to degrade further [30]; hydroxylation of R<sub>1</sub> and Rb<sub>1</sub> were totally consistent with the rat metabolism data [15,28], and hydroxylation of Rg<sub>1</sub> found for the first time provides supporting evidence for the presumption of reference [29]. The results of the present study indicated that metabolism of R<sub>1</sub>, Rg<sub>1</sub> and Rb<sub>1</sub> with zebrafish is practical and feasible.

Metabolism studies with zebrafish have significant advantages of lower cost, far less amount of compound needed, easier set up and higher performance: zebrafish are small, inexpensive to maintain and easily bred in large numbers, and maintenance costs are considerably lower than those for mammals; metabolism experiments using zebrafish can be performed in ordinary laboratories under simple conditions instead of specific animal housing, metabolism cages, and high standard conditions of *in vitro* experiments, *etc.*; compared with sampling blood, bile, fence and urine of mammalian metabolism, sampling solution and fishbody of zebrafish metabolism is much more simple and easier to master, which make zebrafish experiments more efficient with much lower labor intensity. In addition, only small amounts (mg) of compounds are required, about one percent usage of rat metabolism, which make it possible for *in vivo* metabolism study of large number of trace components.

## 3. Experimental

### 3.1. Chemicals and Reagents

Notoginsenoside R<sub>1</sub>, ginsenoside Rg<sub>1</sub>, ginsenoside Rb<sub>1</sub> were purchased from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China), and their purities were all >98%. HPLC grade acetonitrile was purchased from Tedia Company (Fairfield, CT, USA), deionized water was purified by Milli-Q system (Millipore, Bedford, MA, USA), robust purified water, physiological saline (sodium chloride injection) were from Nanjing Xiaoying Pharmaceutical Group Co. Ltd. (Nanjing, China), dimethyl sulfoxide (DMSO) from Sinopharm Chemical Reagent Co. Ltd (Shanghai, China), and the other reagents were of analytical grade.

### 3.2. Animals

The adult zebrafish (*D. rerio*) of mixed sex were provided by Model Animal Research Center of Nanjing University (Nanjing, China), and acclimatized to tap water in a glass aquarium for at least 10 days prior to experimentation. Fish were kept at a temperature of  $25 \pm 1$  °C in a photoperiod of 12:12 h. The fish were fed daily during the acclimatization period, and were fasted overnight before the day of the experiment.

### 3.3. Instruments

A Waters Alliance 2695-ZQ 2000 HPLC-MS system 2695 liquid chromatography system (Waters Corporation, milford, MA, USA) consisting of a quadruple pump, an autosampler, column temperature controller and a PDA detector, Micromass ZQ2000 single-quadrupole mass spectrometer (Waters) with an electrospray ionization source, Masslynx 4.0 ChemStation software; Mettler Toledo AB135-S Analytical Balance (Mettler Toledo, schwerzenbach, Switzerland); KQ3200DE Digital Ultrasonic Washer (Kunshan Ultrasonic Instruments Co. Ltd, Kunshan, China); Labconco Freezer Dryer (Labconco, kansas, MO, USA); TGL-16G Desk Centrifuge (Shanghai Anting Scientific Instrument Factory, Shanghai, China), Organomation N-EVAP<sup>TM</sup> 112 Nitrogen Evaporator (Organomation Associates, Inc. berlin, MA, USA).

### 3.4. Biological Sample Collection

Adult zebrafish were divided into four experimental groups of five fish each after fasting for 12 h one blank control group was exposed to 1% DMSO purified water (blank zebrafish group), three groups were exposed to 30 mL solution of R<sub>1</sub> (9.63 µg/mL), Rg<sub>1</sub> (16.75 µg/mL) and Rb<sub>1</sub> (21.24 µg/mL) in 1% DMSO purified water (drug zebrafish groups), respectively. In addition, the above solutions of R<sub>1</sub> (9.63 µg/ml), Rg<sub>1</sub> (16.75 µg/mL) and Rb<sub>1</sub> (21.24 µg/mL) without zebrafish were used as blank drug controls. Zebrafish of the blank zebrafish group and drug zebrafish groups were sampled at 24 h, respectively, and the zebrafish body samples of each group were combined and washed rapidly with 1% DMSO purified water three times and stored at  $-70$  °C prior to analysis; Solution of blank zebrafish group and drug zebrafish groups at 24 h were combined, respectively, 8 mL (n = 3) of solution of each group were sampled and also stored at  $-70$  °C prior to analysis. Blank drug control solution (8 mL, n = 3) were sampled as above at 0 h, 24 h.

### 3.5. Sample Preparation

The solution sample (8 mL) was freeze-dried to dryness, and the residue was dissolved in 1 mL 90% methanol. After centrifugation at 15,000 rpm for 15 min, 20 µL of the supernatant was introduced into the HPLC-MS system for analysis. The zebrafish body sample (five fish of each group) were cut with scissor, and 1 g was sampled and homogenized with physiological saline (5 mL), followed by centrifugation at 3,500 rpm for 15 min, the supernatant was suspended with methanol (20 mL), and vortex mixed, followed by centrifugation at 3,500 rpm for 15 min. The supernatant was evaporated to dryness with nitrogen at 40 °C, and the residue was dissolved in 90% methanol with the final content

of 1 g zebrafish/mL. After centrifugation at 15,000 rpm for 10 min, 20  $\mu$ L of the supernatant was injected into the HP LC-MS system for analysis.

### 3.6. Analysis Condition

HPLC–MS was performed with a Waters Alliance 2695 - ZQ 2000 single - quadrupole mass spectrometer equipped with an electrospray ionization source. The HPLC analysis was carried out on the column configuration consisted of an Agilent Zorbax Extend reversed - phase C<sub>18</sub> column (5  $\mu$ m, 250 mm  $\times$  4.6 mm) and an Agilent Zorbax extend - C<sub>18</sub> guard column (5  $\mu$ m, 20 mm  $\times$  4 mm). The column was maintained at 30  $^{\circ}$ C, the flow rate was 1.0 mL/min. A gradient elution of 0.05% aqueous formic acid (A) and 0.05% acetonitrile formic acid (B) was used as 7–17% B at 0–10 min, 17–20% B at 10–12 min, 20–21% B at 12–16 min, 21% B at 16–32 min, 21–29% B at 32–40 min, 29–35% B at 40–55 min, 35–65% B at 55–65 min, 65–80% B at 65–80 min, and 80% B at 70–75 min. The mass spectra were recorded with full scan mode in negative mode, capillary voltage 2.5 kV, cone voltage 35 V, drying gas flow rate 320 L/h, ion source temperature 120  $^{\circ}$ C, adjuvant gas temperature 310  $^{\circ}$ C, mass range 100~1200 *m/z*, extract ion current (TIC): [M–H]<sup>–</sup>; [M+HCOO]<sup>–</sup>.

## 4. Conclusions

We have demonstrated for the first time the feasibility of the metabolic study of microamounts of notoginsenoside R<sub>1</sub> (R<sub>1</sub>), ginsenoside Rg<sub>1</sub> (Rg<sub>1</sub>) and ginsenoside Rb<sub>1</sub> (Rb<sub>1</sub>), which are components isolated from *Panax notoginseng*, using a zebrafish model, and metabolic information could be nicely identified with HPLC-ESI-MS. The results showed that metabolic products of R<sub>1</sub>, Rg<sub>1</sub> and Rb<sub>1</sub> resulting from deglycosylation and hydroxylation in zebrafish were highly consistent with those from metabolism of mammals, which confirmed our hypothesis that metabolism studies of compounds from TCMs with the proposed zebrafish model is possible and reasonable. With the advantages of lower cost, easier set up and higher performance, the zebrafish metabolic model may become a novel, powerful model for quick predication on drug metabolism, especially for those trace compounds which could greatly enrich our current knowledge of the metabolism models of TCMs.

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## Conflict of Interest

The authors declare no conflict of interest.

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*Sample Availability:* Samples of the compounds notoginsenoside R<sub>1</sub>, ginsenoside Rg<sub>1</sub> and ginsenoside Rb<sub>1</sub> are available from the authors.

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