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Gas chromatographic–mass spectrometric determination of 20(*S*)-protopanaxadiol and 20(*S*)-protopanaxatriol for study on human urinary excretion of ginsenosides after ingestion of ginseng preparations

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Abstract

An improved gas chromatographic–mass spectrometric method (GC–MS) with a fast solid-phase extraction on a newly introduced C_{18} microcolumn, was applied to study the urinary excretion of 20(*S*)-protopanaxadiol and 20(*S*)-protopanaxatriol glycosides in man after oral administration of ginseng preparations. Using panaxatriol as internal standard, 20(*S*)-protopanaxadiol and 20(*S*)-protopanaxatriol (the aglycones of ginsenosides) could be determined at a detection level of a few ng per ml urine by GC–MS with selected-ion monitoring after their release from glycosides which occur in urine. The extraction recovery of ginsenosides from urine was more than 80% and the intra-assay coefficient of variation was less than 5.0%. The results after intake of single doses of ginseng preparations demonstrated a linear relation between the amounts of ginsenosides consumed and the 20(*S*)-protopanaxatriol glycosides excreted in urine. About 1.2% of the dose was recovered in five days.

Keywords: 20(*S*)-Protopanaxadiol; 20(*S*)-Protopanaxatriol; Ginsenosides

1. Introduction

Ginsenosides are the main biologically active constituents of ginseng (the roots of *Panax ginseng* C.A. Meyer) [1–3]. Ginseng preparations are taken orally as health products or natural remedies for a long time. However, almost all studies concerning absorption, distribution, excretion and metabolism of ginsenosides have been performed on animals with use of huge oral doses of isolated individual ginsenosides [4–8]. So far there is no information on the degree of absorption and excretion of ginsenosides in humans after consumption of ginseng or ginseng preparations.

Analysis of intact ginsenosides in biological fluids is difficult since there are more than 20 structurally similar ginsenosides (ginseng saponins) in ginseng preparations. Furthermore, the parent components may also be transformed to their prosapogenins or sapogenins by glycosidic hydrolysis as well as other

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reactions in the human digestive tract as suggested by the results from animals experiments [9–11] and in vitro experiments [12]. After oral administration of ginsenoside-Rg_1 or Rb_1 (100 mg/kg) to rats, Odani et al. claimed the oral bioavailability of Rg_1 to be 1.9% and that of Rb_1 to be only 0.11%, whereas the cumulative urinary excretions of Rg_1 and Rb_1 were 0.4% and 0.05% of the oral dose, respectively [5,6]. It is evident that a very low excretion of each individual ginsenoside can be expected after intake of ginseng preparations at recommended doses, corresponding to less than 1 mg total ginsenosides intake per kg human body weight.

Methods used for the detection of ginsenosides in animal biological fluids include thin layer chromatography [13], high-performance liquid chromatography [14] and gas chromatography [15]. These methods have a detection limit higher than 0.1 μg/ml which is insufficient for the determination of ginsenosides in human biological fluids. However, using a gas chromatographic–mass spectrometric (GC–MS) assay with selected-ion monitoring based on the determination of the genuine aglycones of ginsenosides, 20(*S*)-protopanaxadiol (*S*-ppd) and 20(*S*)-protopanaxatriol (*S*-ppt), we have recently been able to quantify the concentrations of *S*-ppd and *S*-ppt glycosides in urine samples from Swedish athletes consuming different ginseng preparations [16].

In the present study, the GC–MS method was further improved by replacing conventional Sep-Pak C_{18} cartridges with new C_{18} microcolumns for solid-phase extraction, and this improved method was evaluated by examination of extraction recovery, detection limit as well as assay coefficient variation. With this method, human urinary excretion of *S*-ppd and *S*-ppt glycosides was measured in a pilot study after oral administration of commercial ginseng preparations.

2. Experimental

2.1. Chemicals

Standard ginsenoside-Rb_1 (Rb_1), -Rg_1 (Rg_1) and panaxatriol (PT, as internal standard) were obtained from the Chinese National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). SPEC.VC. C_{18} microcolumns (sorbent weight 15 mg) were purchased from SPEC Division, ANSYS, USA. Sep-Pak C_{18} cartridges were from Waters (Milford, MA, USA). N,N-Bis-trimethylsilyl-trifluoroacetamide (BSTFA) and trimethylsilylimidazol (TMSI) were obtained from Macherey-Nagel (Düren, Germany) and trimethylchlorosilane (TMSCl) from Fluka (Buchs, Switzerland). All other chemicals used were of analytical purity. The stock and working solutions of standards and internal standard were prepared in methanol in concentrations of 1 mg or 10 μg/ml, respectively, and were then stored at 4°C. Urine standard calibrators were prepared by adding aliquots of the working solution (10 μg/ml of Rb_1 and Rg_1) to blank urine. The final concentrations were 1.5 to 105 ng of Rg_1 and 3 to 42 ng of Rb_1 per ml of urine.

2.2. Preparations used

Five commercial ginseng (white ginseng, red ginseng and ginseng extracts) preparations were purchased from pharmacies in Spain, France and Sweden. The *S*-ppd and *S*-ppt ginsenosides in these preparations were identified and quantified by GC and GC–MS methods [16]. The preparations were taken orally by 4 healthy volunteers. Single dose (1.5 to 8.8 mg of *S*-ppt ginsenosides in the preparations) or repetitive doses of a fixed dose (1.5 to 5.8 mg of *S*-ppt ginsenosides and 4.2 to 14.4 mg of *S*-ppd ginsenosides once daily) during 5 to 14 days were used. At least a 10-day washout period was allowed between administrations. This study was approved by the ethical committee at Huddinge hospital, Karolinska institute.

2.3. Urine collection

Following ginseng administration, urine was collected in 24-h samples after a single dose or repetitive daily doses. Some urine samples were collected for shorter periods of the time during the first four days to allow estimation of the excretion rate of *S*-ppt glycosides after taking different preparations. Urine was stored at 4°C until analysed.

2.4. Sample preparation

2.4.1. Solid-phase extraction

To 5–10 ml of urine in a centrifuge tube, 10 μl of internal standard solution containing 10 ng of panaxatriol was added. After centrifugation at 490 *g* for 2 min, the liquid was applied to a SPEC.VC. C_{18} microcolumn previously conditioned with 1 ml MeOH and 1 ml H_2O. The microcolumn (disc) was purified with 1.5 ml H_2O and 1.5 ml of 25% aqueous MeOH. The disc was then dried in vacuum for 5 min. The *S*-ppd and *S*-ppt glycosides absorbed on the disc were eluted with 1.5 ml MeOH and the fraction was collected in a 13-ml test tube with a Teflon-lined cap. In order to compare the extraction recoveries, the urine samples were also applied to the previously used Sep-Pak C_{18} columns for solid-phase extraction of *S*-ppd and *S*-ppt glycosides [16]. With this column, 3 ml MeOH and 5 ml of water were used for washing the column. After applying the sample, the column was purified with 10 ml of water and 10 ml of 25% MeOH–water. Finally, the glycoside fraction was eluted with 5 ml of MeOH.

2.4.2. Oxidative cleavage and derivatization

The last-mentioned MeOH fraction was evaporated and the residue was then dissolved in 2.5 ml of *n*-butanol. After adding 70 mg NaOMe to the solution, the oxidative cleavage of *S*-ppd and *S*-ppt glycosides was carried out at 90°C for 8 h. After centrifugation at 490 *g* for 5 min, the butanol phase was washed once with 1.4 ml of distilled water. An aliquot (about 0.5 ml) of the organic phase containing *S*-ppd and *S*-ppt was taken to dryness followed by trimethylsilylation with 40 μl of a mixed reagent (BSTFA–TMSI–TMSCl, 3:3:2, v/v). The test solution was submitted to GC–MS determination. The blank urine, calibration urine and urine samples were prepared in parallel.

2.5. GC–MS determination

The GC–MS analysis was performed with an Hewlett-Packard 5890II gas chromatograph interfaced to an HP 5989A mass spectrometer (operating at 70 eV), equipped with a HP-7673A autosampler. Ion source and injector temperatures were 250°C and 260°C, respectively. A HP-1 fused-silica capillary column (Ultra performance, 12 m×0.2 mm ID, 0.33 μm film thickness) was used. The carrier gas was helium at a head pressure of 70 kPa and the operating conditions were as follows: The initial temperature was 200°C for 1 min, which was then increased with a rate of 15°C per min to 280°C, and increased finally to 300°C (at a rate of 10°C per min), which temperature was kept constant for 8 min.

Of the test solution 2 μl was injected using a split mode. The fragment ion at *m/z* 199 was used to quantify the trimethylsilylated *S*-ppd and *S*-ppt while the ion at *m/z* 127 was chosen for the trimethylsilylated derivative of the internal standard. Concentrations of the *S*-ppd and *S*-ppt glycosides in urine samples were obtained by measuring the peak area ratios of *S*-ppt (or *S*-ppd) and the internal standard by use of standard curves prepared from Rg_1 and Rb_1.

3. Results and discussion

3.1. Analytical method

This analytical method for study of the urinary excretion of ginsenosides in humans included the following procedures: (1) solid-phase extraction of *S*-ppd and *S*-ppt glycosides from urine with C_{18} microcolumns; (2) oxidative cleavage of glycosidic bonds of the glycosides in alkaline butanol; (3) trimethylsilylation of the cleavage products; (4) GC–MS determination of *S*-ppd and *S*-ppt trimethylsilyl ethers.

3.1.1. Recovery of the extraction

The calibration urine samples at two set concentrations of Rg_1 (20, 60 ng/ml) and Rb_1 (14, 42 ng/ml) with panaxatriol (PT) as internal standard were applied to both the Sep-Pak C_{18} columns and SPEC.VC. C_{18} microcolumns. Comparison of the results obtained with and without using solid-phase extraction, the extraction recoveries of Rg_1 and Rb_1 from urine ($n=5$) with the two columns were about 80% for Rg_1 and higher than 95% for Rb_1 (Table 1). Although both columns provided adequate recoveries, the latter one required much less solvent and time for evaporation of solvent and thus allowed a more rapid analysis.

Table 1
Comparison of extraction recovery of ginsenoside Rb_1 and Rg_1 spiked control urine

Ginsenoside	Rb_1		Rg_1	
	Concentration (ng/ml)	Recovery[a] (%)	Concentration (ng/ml)	Recovery[a] (%)
Sep-pak C_{18} cartridge	14	95±5	20	78±3
	42	98±4	60	81±3
SPEC.VC. C_{18}[b] microcolumn	14	97±4	20	80±3
	42	99±4	60	83±3

[a] Each value represents the mean±S.D of 5 experiments.
[b] Brand name of the microcolumn.

3.1.2. Oxidative cleavage procedure

As we previously described [17], the alkaline cleavage of glycosidic bonds of ginsenosides is oxygen dependent. Therefore oxygen or air atmosphere is necessary for this reaction. But the difference between air and oxygen is negligible when the ginsenosides are below 100 ng per ml urine. The reproducibility of the cleavage procedure with amounts of 100 and 1000 ng of Rg_1 and Rb_1 (corresponding to 10 and 100 ng per ml urine) for each concentration ($n=5$) was below 2.3%.

3.1.3. Standard curves, assay precision and detection limit

When the standard urine spiked with standard ginsenosides Rb_1 and Rg_1 (the main *S*-ppd and *S*-ppt ginsenosides in ginseng) was analysed by GC–MS in the SIM mode, there was a linear relation of the peak-area ratios (*S*-ppd/PT or *S*-ppt/PT) and the concentrations of ginsenosides in the range 3 to 42 ng/ml for Rb_1 and 1.5 to 105 ng/ml for Rg_1 with a coefficient of correlation >0.99. The coefficient of variation ($n=8$) for the intra-assay of Rg_1 and Rb_1 was 4.6% and 5.0% respectively. The quantification limit was 3 ng per ml urine for Rb_1 and 1.5 ng per ml urine for Rg_1 (at a signal-to-noise ratio of 4:1). At this nanogram detection level, it was possible to study the urinary excretion of ginsenosides in humans.

3.2. Analytes

A typical GC–MS chromatogram of aglycones obtained from a human urine sample is shown in Fig. 1 and there are no interfering peaks with the same retention times compared with blank urine samples.

The *S*-ppd and *S*-ppt were identified by comparison of the retention times and complete mass spectra of their trimethylsilyzed derivatives with those of authentic standards. The base peak ion of *m/z* 199 of the TMS-derivatives of *S*-ppd and *S*-ppt, was used for monitoring in order to receive high sensitivity and selectivity.

The amounts of *S*-ppd and *S*-ppt obtained without using the alkaline cleavage procedure were only 5% of those obtained with the whole procedure, suggesting that most of the *S*-ppd and *S*-ppt containing compounds in urine contained one or more sugars attached to the aglycone moiety (absorbed and excreted as ginsenosides or their prosapogines). Although 24,25-hydrated prosapogenins of *S*-ppt ginsenosides have been found in the animal gastrointestinal tract as decomposition and/or metabolites of ginsenosides [9], no such hydrated compounds could

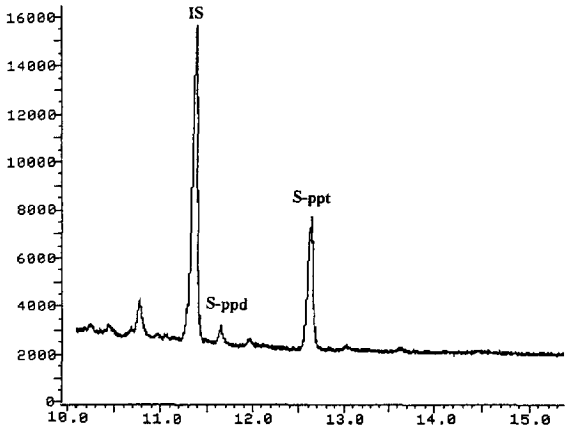

Fig. 1. GC–MS chromatogram of the aglycones of ginsenosides from a urine sample of a volunteer taking ginseng orally. Peaks: *S*-ppd=20(*S*)-protopanaxadiol; *S*-ppt=20(*S*)-protopanaxatriol; I.S.=panaxatriol as internal standard.

be detected in human urine when preparations lacking the hydrated compounds were taken. However, they did occur in the urine in significant amounts when a liquid formulation of ginseng, which contained 24,25-hydrated compounds, had been administered (Fig. 2). The results indicate that these 24,25-hydrated compounds of ginsenosides can be absorbed and excreted by man, and that side-chain hydration of the aglycone moiety is not a prominent feature in ginsenoside metabolism in man.

3.3. Urinary excretion

After the three different ginseng preparations had been orally consumed by the same subject at single doses (4.0–4.5 mg of *S*-ppt ginsenosides), *S*-ppt was detected in urine after 2 h and reached a peak excretion rate after 20 h. The relationship between urinary excretion rates of *S*-ppt glycosides and the midpoint time of the urine collection interval is shown in Fig. 3. A semilogarithmic plot of the excretion rate of *S*-ppt glycosides versus time was found to be linear after the peak excretion rate, with a slope of $-K/2.303$ (presumably a one-compartment model). From the elimination rate constant K, the elimination half-lives ($t_{1/2}$) for these three preparations varied between 13.5 to 17 h.

The results obtained with different single doses (1.5–8.8 mg of *S*-ppt ginsenosides) are shown in Fig. 4. Although different preparations were used, a linear relation was found between the amounts of ginsenosides consumed and the cumulative amounts of *S*-ppt glycosides excreted in the urine. A relatively constant level (about 1.2% of the ingested dose) was obtained during the first 5 days after a single dose.

With repetitive constant doses (once daily), concentrations of *S*-ppd and *S*-ppt glycosides were considerably higher than those found after a single dose, and a steady-state excretion was reached after 5 days. At steady state, the daily recovered amounts of *S*-ppt glycosides from urine appeared to be constant (about 1.5% of the daily dose) (Table 2). The corresponding excretion of the *S*-ppd glycosides was, however, considerably lower and the daily excreted amounts varied from hardly detectable amounts up to 0.2% of the daily dose (Table 2). Thus the amount of *S*-ppt glycosides recovered was at least 5 times that of *S*-ppd glycosides (Table 2). These results are similar to data obtained from animal experiments by Odani et al. [5,6]. The different recoveries may be due to differences in absorption, turnover or excretion of *S*-ppd and *S*-ppt ginsenosides.

The amount of absorbed drug after oral adminis-

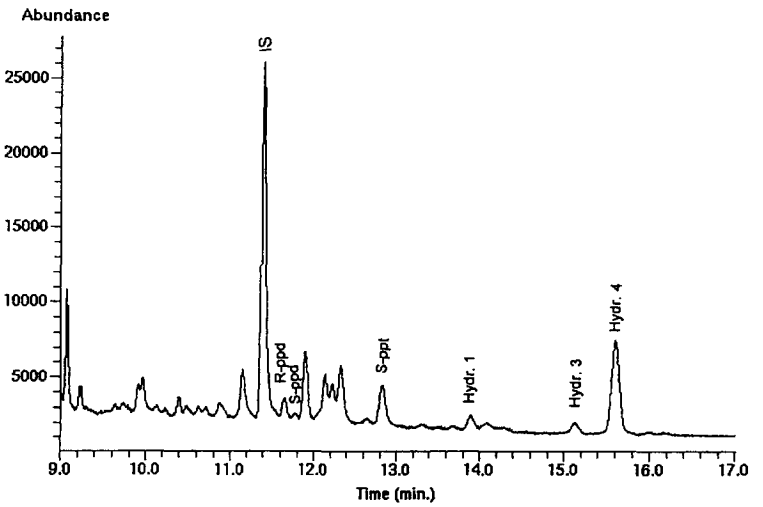

Fig. 2. GC–MS chromatogram of the aglycones of ginsenosides from a urine sample of a volunteer taking a ginseng liquid formulation containing 24,25-hydrated compounds orally. Peaks: I.S.=panaxatriol as internal standard; *R*-ppd=20(*R*)-protopanaxadiol; *S*-ppd=20(*S*)-protopanaxadiol; *S*-ppt=20(*S*)-protopanaxatriol; Hydr.1=24,25-hydrated-20(*R*)-protopanaxadiol; Hydr. 3=24,25-hydrated-20(*R*)-protopanaxatriol; Hydr.4=24,25-hydrated-20(*S*)-protopanaxatriol.

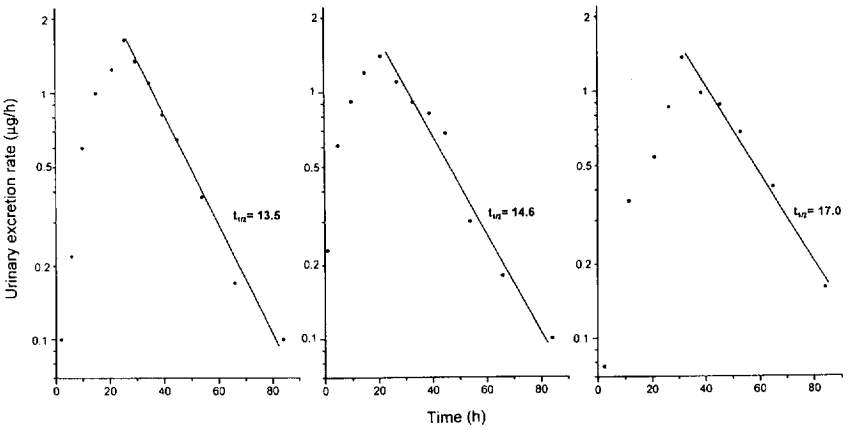

Fig. 3. Urinary excretion time course for *S*-ppt glycosides in a volunteer having received (left) 4.4 mg of *S*-ppt ginsenosides (2 capsules of Active ginseng), (middle) 4.0 mg of *S*-ppt ginsenosides (1 capsule of Arik ginseng), or (right) 4.5 mg of *S*-ppt ginsenosides (3 capsules of Ginsana).

tration may be estimated from the total urinary and biliary excretions of the drug and its metabolites. The relative importance of a biliary excretion of ginsenosides in man is not known. From our preliminary results on urinary excretion of ginsenosides, we conclude that at least 1.2% of *S*-ppt ginsenosides are absorbed from the human gastro-intestinal tract after oral administration of ginseng preparations and that a steady-state excretion is obtained after 5 days. When the steady-state excretion was reached, the daily urinary recoveries of *S*-ppt glycosides were about 1.5% of the daily dose, whereas those of *S*-ppd glycosides were less than 0.24% of the daily dose.

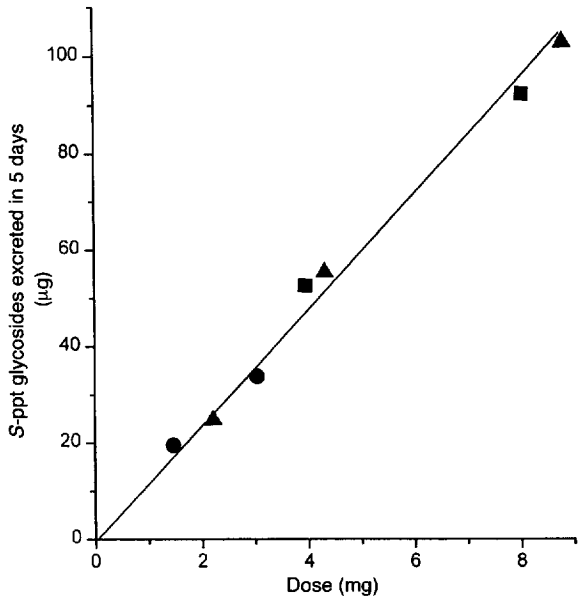

Fig. 4. Relationship between cumulative urinary excretion of *S*-ppt glycosides and comsumption of *S*-ppt ginsenosides in a volunteer after single doses of the following ginseng preparations: Ginsana (●), Active ginseng (▲) or Arik ginseng (■).

In conclusion, the present GC–MS method developed for quantification of *S*-ppd and *S*-ppt glycosides in human urine has a much higher sensitivity than reported methods [13–15] previously used for individual ginsenosides in animal biological fluids. It was thus possible to estimate urinary excretion of ginsenosides after oral administration of ginseng preparations. Furthermore not only *S*-ppd and *S*-ppt, the genuine aglycones of ginsenosides, but also the artificial aglycones such as their 20*R*-epimers as well as their 24,25-hydrated compounds could be detected simultaneously.

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Table 2
Accumulated urinary excretion of 20(*S*)-protopanaxadiol (*S*-ppd) and 20(*S*)-protopanaxatriol (*S*-ppt) glycosides after repetitive dosing of ginseng preparations at recommended dose to three volunteers (A to C)

Preparation[a] and dose once daily	Ginsenosides[b] daily taken		Volunteer	Daily recovered glycosides (% daily dose) at steady rate		
	S-ppd	*S*-ppt		*S*-ppd	*S*-ppt	
Active ginseng capsule (200 mg ginseng powder) 2 capsules	11.8	4.4	A	0.15	1.63	after 5 days
			B	0.24	1.43	after 7 days
Arik ginseng capsule (235 mg red ginseng powder) 1 capsule	14.4	4.0	A	0.21	1.48	after 7 days
Ginsana capsule (100 mg G115 ginseng extract) 2 capsules	7.0	3.0	A	0.08	1.54	after 5 days
			B	0.10	1.35	after 7 days
Bodymax tablet (200 mg ginseng extract) 2 tablets	12.6	5.8	A	0.05	1.32	after 5 days
Royal gerimax capsule (40 mg G120 ginseng extract) 2 capsules	4.2	2.0	C	0.05	1.49	after 7 days
			C	0.05	1.44	after 14 days

[a] Active ginseng: Longli Health Products, Fushun, China, imported to Spain; Arik ginseng: product of Arik Ginseng Int. Cannes, France; Ginsana: product of Pharmaton S.A. (Lugano, Switzerland), sold in Sweden; Bodymax: product of Dansk Droge A/S, Denmark; Royal Gerimax: product of Vitamex, Sweden.
[b] *S*-ppd=20(*S*)-protopanaxadiol; *S*-ppt=20(*S*)-protopanaxatriol.

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