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ORIGINAL ARTICLE

Pharmacokinetics of the citrus flavanone aglycones hesperetin and naringenin after single oral administration in human subjects

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Background and objective: Hesperetin and naringenin, the aglycones of the flavanone glycosides hesperidin and naringin, occur naturally in citrus fruits. They exert interesting pharmacological properties such as antioxidant, anti-inflammatory, blood lipid and cholesterol lowering and are considered to contribute to health benefits in humans. However, no information is available on the pharmacokinetics of the citrus flavanones hesperetin and naringenin after their oral administration to humans as pure aglycones. Therefore, the objective of the present investigation was the evaluation of the pharmacokinetic parameters of hesperetin and naringenin in plasma and urine, after their single oral administration in humans in the form of solid dispersion capsules, and also to improve the absorption rate of flavanones by using aglycones rather than the naturally occurring glycosides.
Design: Six healthy volunteers received orally 135 mg of each compound, hesperetin and naringenin, under fasting conditions. Blood samples were collected at 14 different time points over a 12 h period. Urine was collected over 24 h, in five sequential timed intervals. Plasma and urine hesperetin and naringenin concentrations, after enzymatic hydrolysis of their conjugated forms, were measured using validated high-pressure liquid chromatography methods. Pharmacokinetic parameters for hesperetin and naringenin, such as C_{max}, T_{max}, AUC_{0-t}, $AUC_{0-\infty}$, CL/*F*, *V*/*F*, $t_{1/2}$, MRT, A_e, $A_e(_{0-24})$, and R_{max} were calculated from their plasma or urine concentrations.
Results: Pharmacokinetic analysis showed that both hesperetin and naringenin were rapidly absorbed and their concentrations in plasma observed 20 min after dosing and reached a peak in 4.0 and 3.5 h, respectively. The mean peak plasma concentration (C_{max}) for hesperetin and naringenin were 825.78 ± 410.63 ng/ml (2731.8 ± 1358.4 nmol/l) and 2009.51 ± 770.82 ng/ml (7386.6 ± 2833.4 nmol/l), respectively and the mean $AUC_{0-\infty}$ values were 4846.20 ± 1675.99 ng h/ml and 9424.52 ± 2960.52 ng h/ml for hesperetin and naringenin, respectively. The elimination half-life for hesperetin was found to be 3.05 ± 0.91 h and for naringenin 2.31 ± 0.40 h, respectively. The mean values of the relative cumulative urinary excretion, as percentage of the administered dose, for hesperetin and naringenin, were found to be 3.26 ± 0.44 and 5.81 ± 0.81%, respectively.
Conclusions: Oral administration of the flavanone aglycones, hesperetin and naringenin, lead to their rapid absorption as their conjugated forms. The cumulative urinary recovery data indicated low bioavailability for both flavanone aglycones, owing to extensive first-pass metabolism partly by cleavage of the C-ring by the enzymes of intestinal bacteria leading to degradation products such as phenolic acids.
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Introduction

Flavonoids are polyphenolic plant secondary metabolites ubiquitous in foods of plant origin (Havsteen, 1983). They occur naturally as glycosides and consist of flavones, flavonols, flavanones and isoflavones (Rice-Evans *et al.*, 1996). Hesperidin and naringin are the main flavanone glycosides naturally occurring in citrus fruits (Rouseff *et al.*,

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1987; Kanaze *et al*., 2003). They exert antioxidant (Rice-Evans *et al*., 1996; Miyake *et al*., 1998; Franke *et al*., 2005), anti-inflammatory (Crespo *et al*., 1999), blood lipid and cholesterol-lowering (Montforte *et al*., 1995; Bok *et al*., 1999; Lee *et al*., 1999; Santos *et al*., 1999) and anticarcinogenic activities (Tanaka *et al*., 1997; Yang *et al*., 1997; Berkarda *et al*., 1998). Epidemiological studies indicate an association between the intake of citrus fruits and juices and the risk of ischemic stroke (Joshipura *et al*., 1999). They also alter the pharmacokinetics of a variety of clinically used drugs resulting in drug interactions by inhibiting selected cytochrome *P*-450 enzymes, such as *CYP1A2* and *CYP3A4* (Ghosal *et al*., 1996).

The flavanone glycosides hesperidin and naringin are both rutinosides, that is, the aglycone is linked to glucose and rhamnose sugars at position 7. Flavonoid glycosides, bearing rutinose groups, are hydrolyzed only in the distal part of the intestine and the colon by colonic bacteria, in contrast to flavonoid glucosides, bearing a glucose moiety, which are hydrolyzed already in the small intestine by beta-glucosidases. The released aglycones, hesperetin and naringenin, are recovered in plasma as glucuronides and sulphoglucuronides (Hacket *et al*., 1979; Fuhr and Kummert, 1995; Jang and Kim, 1996; Choudhury *et al*., 1999; Felgines *et al*., 2000; Nielsen *et al*., 2006). Different studies indicate that the aglycone release is the rate-limiting step for their absorption (Manach *et al*., 2003). The flavanone aglycones mentioned also undergo conversion into phenolic acids by means of the cleavage of the C-ring by enzymes of the intestinal microflora (Kim *et al*., 1998). The conjugated flavanone aglycones are excreted in urine (Lee and Reidenberg, 1998; Erlund *et al*., 2001). Moreover, an enterohepatic cycling seems to take place (Manach *et al*., 2003).

To the best of our knowledge, no information is available on the bioavailability and pharmacokinetics of flavanones in humans when provided as pure aglycones. Therefore, the objective of the present investigation was to evaluate the pharmacokinetic parameters of hesperetin and naringenin in plasma and urine, after their single oral dose to healthy subjects in the form of solid dispersion system formulations (Kanaze *et al*., 2006a, b) and also to improve the absorption rate of flavanones by using aglycones rather than the naturally occurring glycosides.

Materials and methods

Chemicals

Hesperetin ((+)-3′,5,7-trihydroxy-4′-methoxyflavanone), 95%, naringenin ((±)-4′,5,7-trihydroxyflavanone), 95%, internal standard 7-ethoxycoumarin and β-glucuronidase/sulphatase (aqueous solution from *Helix pomatia*, Type HP-2, G7017) were purchased from Sigma (St Louis, MO, USA). High-pressure liquid chromatography (HPLC)-grade methanol, acetonitrile and acetic acid were obtained from Merck (Darmstadt, Germany). Bakerbond C18 cartridges, 3-ml 500 mg, were supplied from JT Baker (Deventer, The Netherlands). All other chemicals and solvents used were of analytical grade.

Subjects

The study population included six healthy adult volunteers (five males, one female). All subjects were in good health as assessed by medical history, clinical examination, blood pressure and routine laboratory examinations. The subjects were instructed to abstain from taking any medication including over-the-counter drugs for at least 7 days prior or during the course of the study period and avoiding alcohol or xanthine-containing foods and beverages 36 h before, or during the course of the study, in order to avoid possible drug–drug interactions leading to increased variability of the pharmacokinetic parameters. The subjects have also been requested not to consume foods that contain citrus flavanones (e.g. citrus fruits in any form, tomatoes and tomato sauce and paste, etc.) and they were given a list of prohibited foods.

All subjects were given a detailed description of the study and their informed consent was obtained. The study was performed in accordance with the guidelines of the revised Declaration of Helsinki on biomedical research involving subjects and the requirements of Good Clinical Practice.

Study design and sampling

After an overnight fast of at least 10 h, each subject received a single oral dose (capsule) of 135 mg of each flavanone aglycone, hesperetin and naringenin, along with 240 ml of water. No food was allowed until a standardized flavanone-free meal, consisting of white bread, chicken, rice, salad with vinaigrette dressing, and water, was served 4 h after dosing. Blood pressure and pulse were checked before, during and after the end of the study.

A vein-probe was implemented to each volunteer and blood samples (5 ml) were drawn from each subject into heparinized test tubes immediately before (0) and at 20, 35, 50, 70, 100, 150 min and at 3, 4, 5, 6, 8, 10 and 12 h. Blood samples were centrifuged at 4°C at 3500 *g* for 20 min, and plasma was separated and kept frozen at −20°C in coded polypropylene tubes pending analysis.

Urine was collected in five sequential intervals: 0–3, 3–6, 6–9, 9–12 and 12–24 h after drug administration. The volume of each fraction was measured and an aliquot of 10 ml was transferred into polypropylene tubes and stored frozen at −20°C until analysis.

Quantitative analysis of hesperetin and naringenin in plasma and urine samples

Quantitative analysis of hesperetin and naringenin in plasma and urine samples was conducted using previously reported validated HPLC methods (Kanaze *et al*., 2004a, b). In

brief, plasma and urine samples containing 7-ethoxycoumarin as internal standard were incubated with β-glucuronidase (4000 U) and sulphatase (300 U) (aqueous solution from *Helix pomatia*, Type H-2, Sigma G7017) for 18 h at 37°C, in order to hydrolyze the conjugated forms of hesperetin and naringenin, followed by solid phase extraction using C18 cartridges. The chromatographic separation of hesperetin, naringenin and internal standard was achieved on a 5-μm C8 analytical column (250 × 4.6 mm i.d.) using a mobile phase consisting of methanol/distilled water/acetic acid (40/58/2, v/v/v). The HPLC system was operated isocratically at a flow rate of 1 ml/min at 45°C and absorbance of the eluent was monitored at 280 nm. Quantification of the unconjugated flavanone aglycones, hesperetin and naringenin, was determined by linear regression analysis of peak height ratios versus concentrations of added hesperetin and naringenin. The calibration curves were linear with a correlation coefficient better than 0.999 and the lower limit of quantification for both hesperetin and naringenin was 10 and 50 ng/ml for 1-ml plasma and urine samples, respectively.

Pharmacokinetic evaluation

The pharmacokinetic parameters of hesperetin and naringenin were estimated using standardized model-independent methods (Gibaldi and Perrier, 1982; Rowland and Tozer, 1995). Calculations were carried out with the Siphar/Win package (Simed, Creteil, France).

The maximum plasma concentration (C_{max}) value and the corresponding time that the latter is marked (T_{max}), were taken directly from the individual plasma data. The elimination rate constant (k) was obtained by means of linear regression analysis of the semi-logarithmic plasma concentration–time curve and the elimination half-life ($t_{1/2}$) was calculated by dividing ln 2 by k. The area under the plasma concentration–time curve from administration to the last observed concentration at time t (AUC_{0-t}) and the area under the first moment of the plasma concentration–time curve from administration to the last observed concentration at time t ($AUMC_{0-t}$) were estimated by the use of the linear trapezoidal method, according to the following equations:

$$AUC_{0-t} = \frac{1}{2}\sum_{i=0}^{n-1}(t_{i+1} - t_i)(C_i + C_{i+1})$$

and

$$AUMC_{0-t} = \frac{1}{2}\sum_{i=0}^{n-1}(t_{i+1} - t_i)(C_i t_i + C_{i+1} t_{i+1})$$

where t_i is the ith time point, C_i is the ith available concentration and n is the number of data points.

The area under the plasma concentration–time curve extrapolated to infinitive time ($AUC_{0-\infty}$) and the area under the first moment of the plasma concentration–time curve extrapolated to infinitive time ($AUMC_{0-\infty}$) were estimated by the following equations:

$$AUC_{0-\infty} = AUC_{0-t} + \frac{C_n}{k}$$

and

$$AUMC_{0-\infty} = AUMC_{0-t} + \frac{C_n t_n}{k} + \frac{C_n}{k^2}$$

where C_n is the last measurable concentration and t_n is the time point of the last measurable concentration.

Both apparent total clearance (CL/F) and apparent volume of distribution (V/F) were calculated from $AUC_{0-\infty}$ data according to $CL/F = D/AUC_{0-\infty}$ and $V/F = (D/AUC_{0-\infty})/k$, where D is the administered dose of hesperetin or naringenin (135 mg). Mean residence time (MRT) was determined using the following equation: $MRT = AUMC_{0-\infty}/AUC_{0-\infty}$.

The amount of hesperetin and naringenin excreted into urine during each collection interval (A_e) was determined by multiplying the urine concentration of the drug by the volume of urine collected for each interval. The cumulative amount of hesperetin and naringenin excreted into urine over the entire period of sample collection (0–24 h) ($A_{e_{0-24}}$) was calculated by adding the amount excreted over each collection interval. The rate of drug excretion during each collection interval (R) was determined by dividing the amount excreted in each collection interval by the sampling interval. The maximum rate of drug excretion (R_{max}) and the time of the maximum excretion rate (t_{max}) were derived as the midpoint of the collection interval during which R_{max} occurred.

Results

All six subjects successfully completed the pharmacokinetic study and none reported undesirable or adverse effects after oral administration of the flavanone aglycones, hesperetin and naringenin. All subjects who participated in the study were discharged in good health. Descriptive statistics of the demographic data are summarized in Table 1.

Previous studies indicated that hesperetin and naringenin are found in human plasma and urine almost exclusively as their conjugated forms, glucuronides and sulphoglucuronides (Manach *et al.*, 2003). In our study, hesperetin and

Table 1 Demographic data of six healthy volunteers (five males, one female)

	Age (years)	*Height (cm)*	*Weight (kg)*
Mean	25.0	176	69.17
s.d.	3.9	7.5	10.91
Min	20	165	55
Max	30	185	80

naringenin were quantitated in plasma and urine after hydrolysis of samples with β-glucuronidase/sulphatase and therefore the results represent total hesperetin and naringenin concentrations. None of the subjects had measurable concentrations of either hesperetin or naringenin in plasma or urine at baseline.

The mean$\pm$s.d. plasma hesperetin and naringenin concentration–time curves are illustrated in Figure 1. The inter-individual variability of the plasma hesperetin and naringenin concentrations was fairly low throughout the study. Both flavanone aglycones were absorbed from the gastrointestinal tract and they were measurable in almost all subjects 20 min following oral administration. Descriptive statistics of the pharmacokinetic parameters in plasma for hesperetin and naringenin are summarized in Table 2.

Figure 2 illustrates the mean$\pm$s.e.m. cumulative urinary excretion data for hesperetin and naringenin. For both flavanone aglycones, urinary excretion started in the 0–3 fraction, the maximum excretion rate (R_{max}) occurred in 4.5 h (in the 3–6 h fraction), and was complete in 24 h. The urinary recoveries for hesperetin and naringenin were 3.26$\pm$0.44 and 5.81$\pm$0.81% of the administered dose, respectively (Table 3).

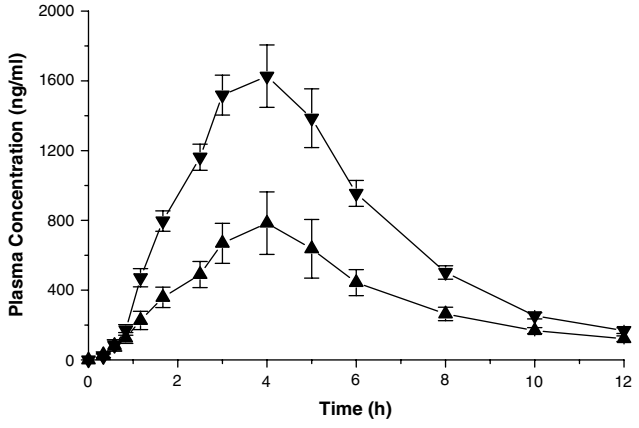

Figure 1 Mean ($\pm$s.e.m.) plasma hesperetin (▲) and naringenin (▼) concentration–time curve following a single oral dose of 135 mg of each compound to six healthy subjects in the form of solid dispersion capsules.

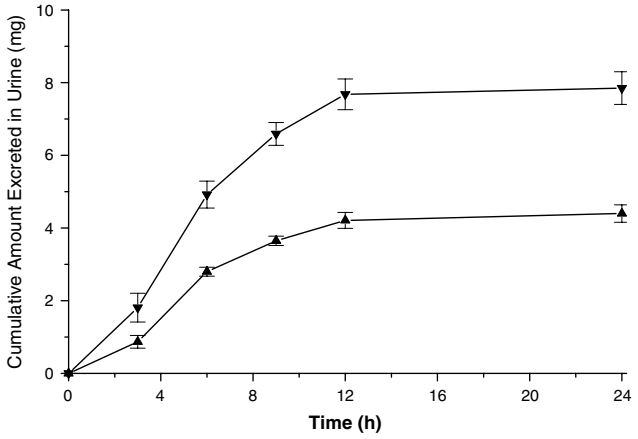

Figure 2 Mean ($\pm$s.e.m.) cumulative urinary excreted amounts (mg), for six volunteers, of hesperetin (▲) and naringenin (▼) and after the administration of a single oral dose of 135 mg of each compound in the form of solid dispersion capsules.

Table 2 Descriptive statistics of pharmacokinetic parameters in plasma of hesperetin and naringenin after single oral administration of 135 mg of each compound to six healthy volunteers in the form of solid dispersion capsules

	$AUC_{0-\infty}$ (ng h/ml)	AUC_{0-t} (ng h/ml)	C_{max} (ng/ml)	T_{max} (h)	CL/F (ml/min)	V/F (l)	$t_{1/2}$ (h)	MRT (h)
Hesperetin								
Median	3837.48	3411.50	623.37	4.00	578.07	143.78	3.12	6.73
Arithmetic mean	4846.20	4300.49	825.78	3.67	497.42	136.38	3.05	6.64
s.d.	1675.99	1697.20	410.63	0.52	138.36	68.40	0.91	1.16
Minimum	3726.94	2988.32	469.38	3.00	296.33	63.69	1.95	5.27
Maximum	7485.36	6837.25	1389.49	4.00	595.17	205.52	3.98	8.25
CV (%)	34.58	39.47	49.73	14.08	27.82	50.15	29.99	17.44
Naringenin								
Median	9190.68	8485.63	1937.87	3.50	241.87	47.37	2.23	5.26
Arithmetic mean	9424.52	8841.51	2009.51	3.67	258.57	51.65	2.31	5.52
s.d.	2960.52	2728.57	770.82	0.82	92.51	19.75	0.40	0.94
Minimum	5274.44	5112.34	1124.26	3.00	168.11	33.15	1.73	4.56
Maximum	13 194.53	12 269.36	3005.20	5.00	420.55	78.89	2.84	7.15
CV (%)	31.41	30.86	38.36	22.27	35.78	38.23	17.54	17.12

Abbreviations: $AUC_{0-\infty}$, area under the plasma concentration–time curve extrapolated to infinitive time; AUC_{0-t}, area under the plasma concentration–time curve from administration to the last observed concentration at time *t*; C_{max}, maximum plasma concentration; CL/F, total clearance; MRT, mean residence time; $t_{1/2}$, elimination half-life; *V*/*F*, volume of distribution.

Table 3 Mean ± s.d. of urinary excretion data of hesperetin and naringenin after administration of a single oral dose of 135 mg of each compound to six healthy subjects in the form of solid dispersion capsules

Time interval (h)	*Mean excreted amount (A_e) (mg)*	*Mean excreted rate (mg/h)*	*Relative urinary excretion (% of the dose)*
Hesperetin			
0–3	0.87 ± 0.43	0.29 ± 0.14	0.64 ± 0.32
3–6	1.93 ± 0.33	0.64 ± 0.11	2.07 ± 0.22
6–9	0.84 ± 0.21	0.28 ± 0.07	2.70 ± 0.23
9–12	0.56 ± 0.30	0.19 ± 0.10	3.12 ± 0.40
12–24	0.19 ± 0.14	0.02 ± 0.01	3.26 ± 0.44
Naringenin			
0–3	1.81 ± 0.97	0.60 ± 0.32	1.34 ± 0.72
3–6	3.11 ± 0.71	1.04 ± 0.24	3.64 ± 0.67
6–9	1.67 ± 0.70	0.56 ± 0.23	4.88 ± 0.57
9–12	1.09 ± 0.43	0.36 ± 0.14	5.69 ± 0.77
12–24	0.17 ± 0.15	0.01 ± 0.01	5.81 ± 0.81

Discussion

The main objective of the present study was to evaluate the plasma and urine pharmacokinetic parameters of hesperetin and naringenin, after their single oral administration in humans in the form of solid dispersion capsules. Secondary objective of the study was also to improve their absorption rate and to decrease the inter-individual variability of the pharmacokinetic parameters concerning the rate and extent of absorption.

Different animal and human studies indicate that the flavanone glycosides hesperidin and naringin, are most likely hydrolyzed by colonic bacteria into their flavanone aglycones, hesperetin and naringenin which are subsequently absorbed into the systematic circulation as glucuronides and sulphoglucuronides (Hacket *et al*., 1979; Bokkenheuser *et al*., 1987; Fuhr and Kummert, 1995; Jang and Kim, 1996; Choudhury *et al*., 1999; Hollman *et al*., 1999; Felgines *et al*., 2000; Erlund *et al*., 2001; Nielsen *et al*., 2006). However, trace amounts of the flavanone glycosides, for example, naringin, can be absorbed intact (Ishii *et al*., 2000). The flavanone glycosides hesperidin and naringin are both rutinosides, and therefore are not hydrolyzed by the beta-glucosidases in the small intestine, but they are hydrolyzed in the distal part of the intestine and the colon by the enteric microflora. Hydrolysis of the flavanone glycosides to their aglycones is probably the rate-limiting step for their absorption (Erlund *et al*., 2001; Manach *et al*., 2003; Nielsen *et al*., 2006). In contrast, the absorption site of the flavonoid aglycones and the flavonoid glucosides, which are hydrolyzed by beta-glucosidases, is the small intestine where they are absorbed much faster (Nielsen *et al*., 2006). Differences in the enteric microflora may be responsible for considerable inter-individual variability regarding the absorption pharmacokinetic parameters (Erlund *et al*., 2001).

The absorption of both hesperetin and naringenin was rapid and much faster than that in previous studies after oral administration of their flavanone glycosides, hesperidin and naringin, either as pure compounds or in the form of citrus juices (Ameer *et al*., 1996; Erlund *et al*., 2001; Manach *et al*., 2003), reflecting the omission of the hydrolytic step. Also, the inter-individual variability of the pharmacokinetic parameters related to absorption, for example, AUC, C_{max} and T_{max}, was fairly low in comparison with that observed in other studies where the flavanone glycosides were administered in the form of citrus juices or as pure compounds (Ameer *et al*., 1996; Erlund *et al*., 2001). Additionally, it is prominent that when citrus flavanones are administered as their aglycones, hesperetin and naringenin, their urinary excretion starts within the first time interval (0–3 h) and reaches their maximum excretion rate in the second fraction (3–6 h), whereas in the case of their administration as flavanone glycosides, their urinary excretion starts fairly later (Erlund *et al*., 2001; Manach *et al*., 2003). It is well known that flavonoid glycosides demonstrate significant transient time before reaching the distal part of the small intestine or the colon where they undergo sugar cleavage and absorption of their aglycones (Ameer *et al*., 1996; Manach *et al*., 2003, Nielsen *et al*., 2006). The values of the elimination half-life ($t_{1/2}$) for hesperetin and naringenin were found to be comparable to those estimated previously, in experiments where the flavanone glycosides were administered in the form of citrus juices (Erlund *et al*. 2001).

The relative cumulative urinary excretion, expressed as percentage of the administered dose, which may be considered as an estimator of the oral bioavailability, was found to be 3.26 and 5.81% for hesperetin and naringenin, respectively, almost comparable than the corresponding values in other studies, where the flavanone glycosides, hesperidin and naringin, were administered as pure compounds (Ameer *et al*., 1996; Ishii *et al*., 2000). However, the cumulative urinary recovery data indicated low bioavailability for both flavanone aglycones, possibly owing to extensive first-pass metabolism partly by cleavage of the C-ring by the enzymes of intestinal bacteria leading to degradation products such as phenolic acids (Booth *et al*., 1958; Felgines *et al*., 2000).

To our knowledge, this is the first report to evaluate the pharmacokinetic parameters in plasma and urine of the flavanone aglycones, hesperetin and naringenin, after their single oral administration in humans in the form of solid dispersion formulations.

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