SHORT AND MID-TERM BIOAVAILABILITY OF FLAVANONES FROM ORANGES IN HUMANS

S. De Pascual-Teresa¹, C. Sánchez-Moreno¹, F. Granado², B. Olmedilla¹, B. De Ancos¹ and M. P. Cano¹

¹Instituto del Frío, CSIC, E-28040-Madrid, Spain, ²Unidad de Vitaminas, Hospital Universitario Puerta de Hierro, E-28035-Madrid, Spain

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ABSTRACT: Flavanones are a group of flavonoids characteristic of citrus fruits that have been associated with hypocholesterolemic and antioxidative effects. Flavanones from oranges, mainly hesperetin and naringenin, have also been proved to be effective in the inhibition of chemically induced carcinogenesis in animal models. Although there are some studies regarding the bioavailability of flavanones from orange juice or pure compounds, the levels of hesperetin and naringenin that may be reached in plasma after the consumption of oranges in a normal diet in humans is largely unknown. The purpose of this study was to investigate the effects of single and repeated intakes of a normal amount of orange flavanones on hesperidin and naringin plasma concentrations and antioxidant activity in humans. After ingestion of 400 g of minimally processed orange fruit the levels of plasma hesperetin increased gradually throughout the study period. The maximum level of hesperetin reached in plasma was 148 nmol/L, whereas for naringenin the maximum level was only 15 nmol/L. Plasma FRAP decreased slightly during the study, however this decrease was not significant. In the multiple-dose-response study the results showed a significant increase over the baseline levels of hesperetin in plasma after 7 and 14 days of daily ingestion of 200 g orange, thus indicating that the effect obtained after a single-dose ingestion is not predictive of the effect after a multiple-dose regimen.

KEYWORDS: Bioavailability, Flavanones, Hesperetin, Naringenin, and Orange.

INTRODUCTION

Flavanones are a group of flavonoids that possess the unique characteristic of being restricted almost exclusively to fruits of the genus Citrus. They are present in nature mainly as the hesperetin and naringenin glycosides. The average intake of flavanones in humans has been estimated to be 36.6 mg/day, including naringenin (8.3 mg/day) and hesperetin (28.3 mg/day) (Kumpulainen et al., 1999). However the individual intake of flavanones may vary enormously from one person to another due to the fact that they are only found in high quantities in citrus fruits and to a much lesser extent in aromatic herbs. Moreover, flavanone intake from orange fruit or orange products may differ depending on the cultivar and amount of albedo ingested, since flavanones are especially rich in this fruit tissue (Tomas-Barberan and Clifford, 2000).

Flavanones are found at their Cmax in plasma approximately 5 hours after ingestion of orange and grapefruit juices or pure compounds (Manach et al., 2005). They are metabolized in the liver and intestine and appear in blood and urine as their aglycons and glucuro- and/or sulfoconjugate forms (Manach et al., 2003). More recently methylation and hydroxylation of naringenin has also been described in rat experiments (Silberberg et al., 2006). The recovery of flavanone metabolites in urine has been estimated to be 8.6% for hesperidin and 8.8% for naringin (Manach et al., 2003). After ingestion of orange and grapefruit juices containing 126 mg of hesperetin equivalents and 199 mg of naringenin equivalents respectively, plasma concentrations reached 2.2 μmol/L for hesperetin metabolites and 6 μmol/L for naringenin metabolites (Erlund et al., 2001).

The potential biological properties of flavanones have been the subject of many studies in the last few years. Orange juice and hesperetin have been shown to inhibit chemically induced carcinogenesis in laboratory animals (Erlund et al., 2004). Naringin has been proved to be a potent inhibitor of the secretion of a potent inducer of angiogenesis, namely the vascular endothelial growth factor, in a breast cancer cell line at physiological concentrations (Schindler and Mentlein, 2006). Flavanones have also been reported to modulate lipid metabolism, to have anti-inflammatory properties and to bind to estrogen receptors (Erlund et al., 2004).

Oranges are among the main sources of flavanones and other antioxidants, such as carotenoids and vitamin C, in the human diet. However, the intake of fresh oranges and fresh fruits in general has decreased in recent years, giving rise to an increase in
the consumption of processed substitutes, such as juices or fruit preserves. Minimal processing of fruits has been shown to be a useful means for increasing fruit consumption, preserving their potential health related attributes while at the same time increasing their convenience (Gil et al., 2006).

Although there are some studies on the bioavailability of flavanones from orange juice, the levels of hesperetin and naringenin that may be found in the plasma after orange fruit consumption within a normal diet in humans have not been systematically studied. Furthermore, to our knowledge, no studies on long or medium term bioavailability of flavanones have been conducted so far. The aim of this work was to investigate the effects of single or repeated intakes of a normal amount of orange flavanones on hesperidin and naringin plasma concentrations and antioxidant activity in humans.

MATERIALS AND METHODS

Chemicals. Hesperetin, naringenin, narirutin, hesperidin, apigenin, β-glucuronidase, sulfatase and 2, 4, 6 tripridyl-s-triazine (TPTZ) were obtained from Sigma (St. Louis, MO). All other reagents were also obtained from Sigma unless otherwise stated and were of analytical or HPLC grade where applicable.

Orange preparation and analysis. Oranges (Citrus sinensis L., Osbeck, ‘Navelina’), (Valencia, Spain) were purchased from a local supermarket. The fruits were selected, washed in sterile water and were of analytical or HPLC grade where applicable. Reagents were also obtained from Sigma unless otherwise stated and were of analytical or HPLC grade where applicable.

Vitamin C and carotenoids were also quantified by HPLC with DAD detection as described in Sánchez-Moreno et al. (2003).

Subjects. Fourteen healthy volunteers (7 men and 7 women) were enrolled in this study. The subjects were aged between 20 and 30 (25 ± 4 y), and their mean body mass index was 24.2 ± 2.7 kg/m² and did not change significantly during the study. All the subjects continued their habitual diets during the study. Subjects were taking no vitamin or mineral supplements and no medications. None of the subjects were pregnant, lactating, or had any chronic illness. All study participants were in good health based on their medical history, a physical examination, and normal clinical laboratory test results, including measurement of triacylglycerol (0.8 ± 0.3 mmol/L), cholesterol (4.1 ± 0.5 mmol/L), glucose, and hematocrit. Subjects received oral and written information about the study and gave their written consent. The study was approved by the Clinic Research Ethics Committee of the Hospital Universitario Puerta de Hierro, Madrid.

Study design. The study was divided into 2 components: a dose-response test and a multiple-dose-response study. For the dose-response test, an intravenous catheter was inserted into each subject’s forearm after he or she had fasted for at least 8 h. After blood samples were collected at baseline, the volunteers consumed 400 g of minimally processed oranges and blood samples were taken every 60 min up to 6.5 h. Blood samples were collected in heparin-coated tubes and were centrifuged at 2000 x g for 15 min at 4 ºC. After the plasma was collected, aliquots were stored at -80 ºC for analysis of the ferric reducing ability of the plasma (FRAP) and flavanone content. For the multiple-dose response study, the subjects were instructed to consume 200 g of orange in the morning, for 2 consecutive weeks. Blood samples were again taken during the intervention on days 7 and 14 of the study after 12 h of fasting and before the daily orange intake.

Plasma analysis. Hesperetin and naringenin were quantified in plasma after enzymatic hydrolysis of their conjugated forms. Ascorbic acid (final concentration 1 mmol/L) and acetic acid (0.65 mmol/L; 40 mL) were added to plasma to avoid degradation. Plasma samples (0.4 mL) were added to 0.5 mL 0.1 M phosphate buffer pH 6.2 with apigenin (180 mmol/L; 100 mL) as internal standard and hydrolyzed with 25U b-glucuronidase and 9U sulfatase at 37ºC for 3 h. Methanol (0.5 mL) was added to terminate the reaction. Acetonitrile (2.5mL) was added to precipitate proteins and extract flavonoids. The samples were vortexed for 30 s every 2 min over a ten minute period, before using a Hewlett-Packard Chem Station and related software. Identification of the flavanones was carried out by HPLC by comparing the retention times and UV-vis absorption spectra with those of the hesperidin, naringin, hesperetin and naringenin standards. Quantification of the flavanones was achieved by the absorbance recorded in the chromatograms relative to the external standards of flavanones previously referred to.
and passed through a 4 mm polyvinylidene-difluoride syringe filter into vials for HPLC analysis. The hydrolysates were analyzed using an Agilent 1100 series liquid chromatograph/mass selective detector equipped with a quadrupole (G1946D) mass spectrometer (Agilent Technologies, Waldrom, Germany). The liquid chromatographic system consisted of a quaternary pump (G1311A), on-line vacuum degasser, autosampler (G1313A) and a thermostatic column compartment, connected in line to a DAD (G1315B) before the mass spectrometer. Separation was achieved with a reversed-phase C-18 Luna (Phenomenex). Elution was performed with a gradient between Milli-Q water at a pH 2.5 (solution A) and acetonitrile (solution B) at a flow rate of 0.5 mL per min and an injection volume of 20 μL in the same conditions as described previously. Electrospray ionization (ESI) in the negative ionization mode was used. The electrospray capillary voltage was set at 2500 V, with a nebulizing gas flow rate of 12 L/min and a drying gas temperature of 150 °C. MS analyses were recorded in the SIM mode at 301 m/z for hesperetin, 271 for naringenin, and 269 for apigenin, as the internal standard. Quantification of the hesperetin and naringenin was performed by integrating the SIM peaks with the integration software incorporated in the Agilent ChemStation.

Ferric reducing ability of plasma (FRAP). The FRAP assay was performed as previously described (Benzie and Strain, 1996). FRAP reagent was freshly prepared each day by mixing together 10 mM 2, 4, 6 tripridyl-s-triazine (TPTZ) and 20 mM iron (III) chloride in 0.25 M acetate buffer, pH 3.6. The absorbance of plasma as compared to ascorbic acid was read at 593 nm (Perkin Elmer UV/Vis Lambda Bio 20) 6 minutes after incubation at room temperature against a blank of FRAP reagent and distilled water. FRAP values are expressed as ascorbic acid equivalents.

**Statistical Analysis.** Statistical analysis was performed by using the SPSS software package for Windows (version 14.0.1, SPSS, Richmond, CA). Values are reported as means ± SEMs unless noted otherwise, and the significance level was set at a p= 0.05.

**RESULTS AND DISCUSSION**

**Orange analysis**

This study was performed using minimally processed oranges prepared at the Instituto del Frio. The analysis of their flavanone content by HPLC showed that their levels of the flavanone glycosides, naringin and hesperidin, in the samples were 15.98 ±1.85 mg/100 g and 49.41±17.72 mg/100 g of fresh fruit, respectively. The free flavanone forms, naringenin and hesperetin, were not detected in any of the samples.

This meant that there was a total ingestion of hesperidin and naringin in the dose-response test of 198 mg and 64 mg respectively. These quantities, corresponding to 400 g orange, are in the same range as those obtained by Manach et al. (2003) and Erlund et al. (2001) by giving the volunteers half a liter of commercial orange juice.

In the case of the multiple-dose-response study, the quantity of orange ingested daily corresponded to 49 mg as hesperetin equivalents and 15 mg as naringenin equivalents. It should be noted that this flavanone intake could be easily achieved with a normal diet. Other antioxidants present in the oranges used in the present study were vitamin C, with a concentration of 42 mg per 100 g and carotenoids, 173 mg per 100 g of fresh fruit.

**Levels of plasma flavanones in the dose-response test**

Plasma levels of hesperetin at the baseline point were 29.2 ± 19.8 nmol/L and undetectable in the case of naringenin. After ingestion of 400 g of minimally processed orange fruit the levels of hesperetin in plasma increased gradually throughout the study. The duration time for the short-term bioavailability study was set at 6.5 hours since it could be taken as the between-meals time in a normal diet. As can be seen in Figure 1, the maximum level of hesperetin reached in plasma after the ingestion of 98 mg of hesperidin equivalents from orange was 148 nmol/L, whereas for naringenin the maximum level was only 15 nmol/L.

**FIGURE 1.** Plasma hesperetin (●) and naringenin (♦) after ingestion of 400 g minimally processed oranges. Results are expressed as mean ± SD

Manach et al (2003) have studied the pharmacokinetics of flavanones from a liquid matrix and they found that the Cmax were reached after between 5.4 and 5.8 hours in the case of hesperetin and after between 4.6 and 5 hours in the case of naringenin. In this work we have studied the postprandial effect of orange consumption in a normal diet on the flavanone plasma levels and the results show that in the case of a solid matrix the levels of both, hesperetin and naringenin, in the plasma of the volunteers reached a maximum after 6.5 hours. The maximum concentrations found in the present study were in accordance with those found in another study for a similar flavanone dose.

As described by Erlund et al. (2001) there are great inter-individual variations in flavonoid bioavailability in general and more concretely in the case of orange flavanones. This variability may be due to differences in the levels of enzymes and receptors involved in the transport of flavonoids through the intestinal wall but also due to different levels of other enzymes, which influence the metabolism, and distribution of flavonoids in humans.
Levels of plasma flavanones in the multiple-dose-response study

As previously stated, the present study had a two-fold objective: firstly, we intended to establish the kinetics of flavanone appearance in plasma during the postprandial period after orange ingestion; and secondly, we aimed at clarifying whether there is any accumulative effect of this group of flavonoids in human plasma after a daily ingestion of 200 g of oranges over periods of 7 and 14 days.

Some authors have already hypothesized that because the half-lives of conjugated flavonoids are long, between 23 and 28 hours, there may be an accumulative effect when flavonoid rich foods are consumed on a regular basis (de Pascual-Teresa et al., 2004; Nijveldt et al., 2001; Young et al., 1999). In this sense Riso et al. (2005) have studied the effect on the basal levels of a daily intake of blood orange juice, containing 21 mg of cyanidin-3-glucoside, for 21 days, measured after overnight fasting. In this work, they have demonstrated that there was an accumulative effect that increased plasma levels of this flavonoid approximately ten-folds with respect to the levels at baseline before the intervention. However, till now there have been no studies on the mid or long-term bioavailability of orange flavanones.

Our results showed in the multiple-dose-response study a significant increase over the baseline levels of hesperetin in plasma after 7 and 14 days of daily ingestion of 200 g orange (Figure 2). On day 0 and after an overnight fast the levels of hesperetin in plasma were 29.2 ± 19.8 nmol/L and undetectable in the case of naringenin. After 7 and 14 days of daily orange consumption the plasma levels of hesperetin increased to 94 and 91 nmol/L respectively. Plasma levels of naringenin increased to 3 nmol/L by day 7 and to 2 nmol/L after 14 days of daily orange intake. These levels of both hesperetin and naringenin in plasma reveal an accumulative effect of orange flavanones when fruits are consumed every day. However hesperetin and naringenin plasma levels remained stable between 7 and 14 days of daily 200 g orange intake showing that a “ceiling effect” may operate, in which the accumulation of flavanones in the plasma reaches a saturation point.

In the medium term, daily intake of orange fruit, in sufficient amounts, may produce an increase in plasma levels of flavanones and their metabolites thus explaining the biological effect that has been shown in epidemiological studies (Erlund et al., 2004).

**Plasma FRAP**

Plasma FRAP at baseline was 527 ± 105 μM ascorbic acid equivalents (Figure 3). It should be noted that the great inter-individual variation in plasma FRAP values encountered in the present study is in agreement with those found by other authors (Fernandez-Pachon et al., 2005; Dragsted, 2004) and represents, on average, a variation coefficient of around 20%. 6.5 hours after ingestion of 400 g of minimally processed orange plasma FRAP decreased by 41 μM ascorbic acid equivalent. Plasma FRAP also decreased slightly over the study period of 7 days (456 ± 85 μM ascorbic acid) and 14 days (477 ± 142 μM ascorbic acid), however this decrease was not statistically significant. In this sense, some authors have shown that ingestion of other antioxidant products such as cherries do decrease plasma FRAP, showing that in the case of cherries this is mainly due to a parallel decrease in urate levels (Jacob et al., 2003). Conversely other authors have shown that consumption of other polyphenol rich products such as apples or wine do increase plasma FRAP and urate levels under similar conditions (Fernandez-Pachon et al., 2005; Lotito and Frei, 2004). As mentioned before, the great inter-individual variation in plasma antioxidant capacity measured as plasma FRAP, may explain, at least in part, our results.

**CONCLUSIONS**

After ingestion of 400 g of minimally processed orange fruit the levels of hesperetin in plasma increased gradually until the end of the study. The maximum level of hesperetin reached in plasma was 148 nmol/L, whereas for naringenin the maximum level was only 15 nmol/L. Maximum plasma levels were reached at the end of the postprandial period as evaluated in the present study and, were higher than steady state plasma levels, for orange intake which is compatible with a balanced diet.

In the multiple-dose-response study the results showed a significant increase over the baseline levels of hesperetin in plasma after 7 days of daily ingestion of 200 g orange. Plasma levels of flavanones remained stable between 7 and 14 days of daily orange intake indicating a saturation effect. No significant changes were
observed in plasma FRAP after 7 or 14 days of orange consumption. These results indicate that orange flavanones may not contribute to the antioxidant response evaluated as plasma FRAP, probably due to the rather low antioxidant power of flavanones.

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CONFLICT OF INTEREST STATEMENT

The authors have no relevant financial interest in this article.

REFERENCES


