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Bioavailability of Apigenin from Apiin-Rich Parsley in Humans

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Abstract

Aim: Absorption and excretion of apigenin after the ingestion of apiin-rich food, i.e. parsley, was tested. ***Methods:*** Eleven healthy subjects (5 women, 6 men) in the age range of 23–41 years and with an average body mass index of 23.9 ± 4.1 kg/m^2 took part in this study. After an apigenin- and luteolin-free diet, a single oral bolus of 2 g blanched parsley (corresponding to 65.8 ± 15.5 μmol apigenin) per kilogram body weight was consumed. Blood samples were taken at 0, 4, 6, 7, 8, 9, 10, 11 and 28 h after parsley consumption and 24-hour urine samples were collected. Apigenin was analyzed in plasma, urine and red blood cells by means of HPLC-ECD. ***Results:*** On average, a maximum apigenin plasma concentration of 127 ± 81 nmol/l was reached after 7.2 ± 1.3 h with a high range of variation between subjects. For all participants, plasma apigenin concentration rose after bolus ingestion and fell within 28 h under the detection limit (2.3 nmol/l). The average apigenin content in 24-hour urine was 144 ± 110 nmol/24 h corresponding to 0.22 ± 0.16% of the ingested dose. The flavone could be detected in red blood cells without showing dose-response characteristics. ***Conclusions:*** A small portion of apigenin provided by food reaches the human circulation and, therefore, may reveal biological effects.

Introduction

Flavonoids are abundant phenolic plant substances that can be divided into six subclasses which all share the common phenylchromane structure consisting of two aromatic rings (A and B) and an oxygenated heterocyclic ring (C) [1]. Apigenin (5,7,4′-trihydroxyflavone) belongs to the subclass of flavones and is present in very low amounts in the human diet [2]. Thus, apigenin has not been examined well for aspects like bioavailability distribution or excretion in humans. Important food sources of apigenin as identified to date are parsley and celery [3]. Only few studies gave indication on the average dietary intake. In men participating in the ATBC Study in Finland (n = 25,041), a mean daily intake of 10 mg flavonols and flavones (quercetin, kaempferol, myricetin, luteolin and apigenin) was estimated and apigenin comprised 0.5% of the total [4]. In Hungarian children (n = 521) and adults (n = 204), average apigenin intake was estimated to be 0.57 ± 0.71 and 0.85 ± 0.87 mg/day, respectively [5].

Potential biological effects of apigenin are described in vitro and in vivo. The substance is suggested to be chemopreventive, a modulator of certain signal transduction pathways [6], an in vivo inhibitor of UVB-induced skin tumor in mice [7], and an inhibitor of several protein kinases and enzymes which may play a major role in tumor promotion (like cyclooxygenase-2). In addition, the substance induces apoptosis in certain human carcinoma cells, e.g. breast, prostate, thyroid, leukemia, melanoma and colon, but not in normal cells [8, 9]. Apigenin is an

estrogenomimetic compound and interacts with P_{450} and phase II enzymes involved in the metabolism of estrogens [10]. Furtheron, apigenin possesses anti-inflammatory, anti-proliferative and free radical-scavenging properties in many in vitro systems [9]. Apigenin is described as non-mutagenic and non-toxic [6].

So far, little is known about the bioavailability of apigenin. One study with a relatively high limit of detection for apigenin found no evidence for apigenin absorption from the human intestinal tract [11]. However, in another study, apigenin was detected in urine samples after parsley consumption [12]. After having developed a very sensitive method for measuring apigenin in plasma [13] we were able to conduct a study on the bioavailability of apigenin from food using a single bolus of parsley *(Petroselinum crispum var. crispum)*. Parsley is high in apiin (apigenin-7-*O*-apiosylglucoside) and has concomitantly a comparably low concentration of other flavonoids [12, 14, 15]. Therefore, this study was conducted to assess the effect of parsley intake on the apigenin concentrations in plasma and urine allowing conclusions on apigenin bioavailability. Additionally, red blood cells (RBC) should also be analyzed for apigenin.

Material and Methods

Study Design

On the day before parsley consumption, a fasting blood sample was taken and the participants had to adhere to an apigenin- and luteolin-free diet. On the experimental day the subjects consumed the parsley meal early in the morning. Afterwards, blood samples were collected 4, 6, 7, 8, 9, 10 and 11 h after parsley consumption (fig. 1). Additional feeding was standardized by means of a formula diet. In addition, 24-hour urine was collected by the study participants. The apigenin-free diet had to be followed for the rest of the experimental day. On the day after, another fasting blood sample was taken.

Subjects and Diet

Five women and 6 men were recruited and all volunteers completed the study. The age of the subjects was 28.8 ± 5.4 years (mean ± SD) with a range of 23–41 years, and the mean body mass index was 23.9 ± 4.1 kg/m^2. The participants declared that they were healthy, no woman was pregnant and no one was suffering from metabolic disorders. All subjects gave written consent after having been informed about the study aims and procedures. The study was conducted according to the Helsinki Declaration.

All volunteers were instructed to avoid apigenin- and luteolin-rich foodstuff throughout the study: cabbage and other cruciferous vegetables, salad, celery, olives, spinach, leek, pepper, onions, garlic, oregano, rosemary, thyme, parsley and grapefruit. Luteolin-rich food has been included in this list for luteolin being the major metabolite of apigenin [10]. The parsley meal consisted of 2 g parsley per kg body weight corresponding to 149.45 ± 35.21 g of parsley

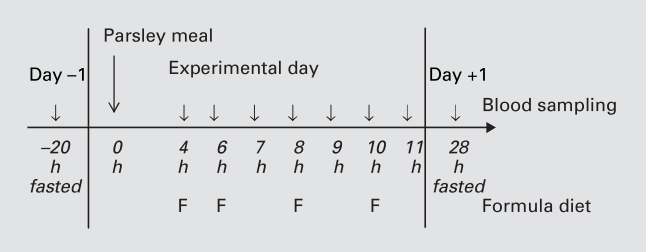

Fig. 1. Time schedule.

(mean ± SD) and providing an average of 17.77 ± 4.19 mg (65.76 ± 15.49 μmol) of apigenin. Parsley (leaves) from the same batch was provided to all subjects. The apigenin content in parsley was 11.89 mg/100 g edible portion. By means of our method [13] we could monitor several other flavonoids in parsley and the amount of kaempferol (3.02 mg/100 g), quercetin (0.02 mg/100 g), isorhamnetin (1.79 mg/100 g), and luteolin (0.31 mg/100 g) was quantified (myricetin was not recordable by this method). Parsley was washed, dried and frozen at –18°C until consumption. On day –1, parsley was blanched in butter (on average 10% of the parsley portion) for 10 min and salted. The content of butter in the parsley meal was on average 14.95 ± 3.52 g. The prepared food was stored at 5–8°C until consumption on the experimental day. As apigenin in parsley is stable to microwave heating [12], the participants were allowed to consume the parsley hot (microwave heating) or cold, with wheat toast/bread, salt, and pepper.

During the experimental day, the subjects were provided with formula diet (Fresubin Orginal Drink, Fresenius Kabi, Bad Homburg, Germany; per 100 ml: 3.8 g protein, 13.8 g carbohydrate, 3.4 g fat) in order to minimize possible food-derived effects. 60% of the estimated daily energy requirements (30 kcal/kg body weight) were given at fixed time points in four portions (fig. 1). The average daily energy requirement was estimated to be 2,680 ± 252 kcal (mean ± SD), thus 1,357 ± 28 kcal (mean ± SD) were consumed via the sip feed supplement. After the last blood sampling on the experimental day, the subjects were free to adhere to the apigenin- and luteolin-free diet.

Sample Collection, Preparation, and Apigenin Analysis

Venous blood samples were collected in EDTA vacuum tubes (S-Monovette®; Sarstedt, Nümbrecht, Germany). Plasma was prepared by centrifugation (4,000 rpm for 15 min), and RBC were washed twice with 0.9% NaCl solution. The volume of the 24-hour urine sample per subject was measured and aliquots were taken. All samples were stored at –80°C until analysis.

Apigenin was measured as aglycone after enzymatic (biological specimens) or acid (parsley) hydrolysis. The method is described in detail elsewhere [13]. Briefly, plasma and urine samples were spiked with fisetin as internal standard. After addition of buffered sulfatase (also containing glucuronidase), samples were incubated for hydrolysis (37°C, 30 min). The reaction was stopped by adding phosphoric acid (85%). Using solid-phase extraction (Oasis HLB; Waters Corp., Mass., USA), apigenin was eluted with 2 ml of methanol. The extract was dried under vacuum and the residue re-dissolved in 100 μl of a mixture of methanol and water (60/40; v/v);

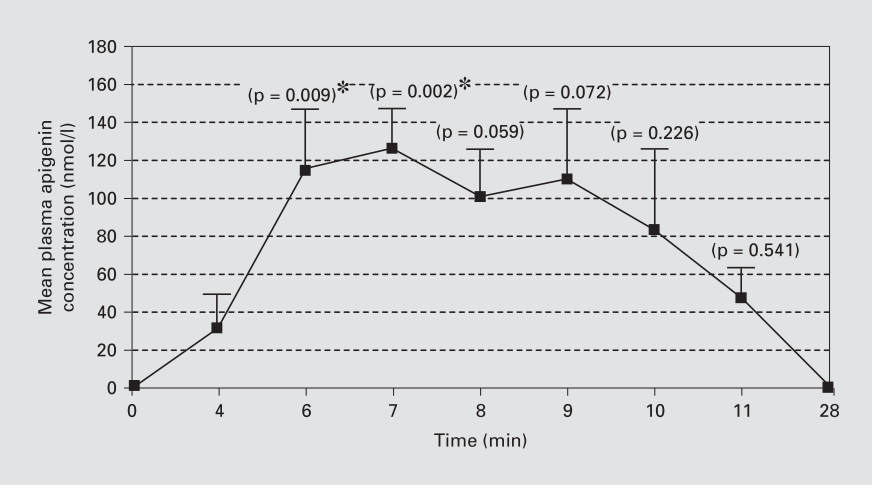

Fig. 2. Mean (±SEM) plasma apigenin concentrations (nmol/l) after parsley bolus. (paired t-test for differences between time points, all compared with 4-hour values; * significant).

30 µl were injected into HPLC system. Ascorbic acid was added to avoid autoxidation. RBC were hemolyzed by addition of distilled water and the buffered samples were dropped into 4 ml of acetone, mixed and centrifuged (5,000 rpm, 5 min). The supernatant was dried under vacuum until the acetone was completely removed.

A modified method of Hertog et al. [16] was used for the measurement of the apigenin concentration in parsley. 5 ml of methanol and 100 µl of fisetin (1.93 µg/ml) were added to 0.5 g of homogenized parsley leaves and the sample was then vortexed. After 30 min of sonication the sample was centrifuged (4°C, 5,000 rpm, 10 min) and 5 ml of hydrochloric acid (2.4 *M*) was added to the supernatant for hydrolysis at 90°C. After cooling down, the sample was again centrifuged and filtered through a 0.45-µm syringe filter.

The HPLC system included a diode array detector (Gynkotek, Germering/Munich, Germany) and an electrochemical detector (ED 40; Dionex, Idstein, Germany). The substances were separated by means of two analytical columns put in row, Luna RP-C18 (4.6 × 150 mm; Phenomenex, Aschaffenburg, Germany) and Zorbax Eclipse XDB-C18 (3.0 × 150 mm; Agilent Technologies, Böblingen, Germany). The electrochemical detector had a measuring voltage of +1,100 mV and the column oven temperature was 60°C. Running time was 120 min with a fixed flow rate of 0.4 ml/min. Two solvents were used, 0.1% phosphoric acid (A) and methanol (B), with the following gradient: 0 min 100/0; 15 min 82/18; 50 min 75/25; 65 min 60/40; 109 min 44/56; 110 min 0/100; 115 min 0/100; 120 min 100/0. Peak identification was ensured by comparison with the retention time of the flavonoid standards and by spectra analysis. Substances were quantified using the internal standard method. The samples were analyzed in duplicate. For apigenin, the limit of detection was 2.3 nmol/l, recovery was 83.1% in plasma and 76.0% in parsley, and the coefficient of variation of 7.4%.

Statistics

Comparison of plasma apigenin concentrations between time points were conducted using paired Student's t test with $p < 0.05$ considered as statistically significant.

Table 1. Plasma apigenin concentration (nmol/l; mean, standard deviation (SD), standard error of mean (SEM), median, minimum and maximum) by time (h) after parsley consumption

Time h	Mean nmol/l	SD nmol/l	SEM nmol/l	Median nmol/l	Min nmol/l	Max nmol/l
0	0.00	0.00	0.00	0.00	0.00	0.00
4	31.02	43.73	13.18	13.02	2.87	125.96
6	115.27	102.28	30.84	57.57	26.63	307.80
7	126.62	80.53	24.28	115.80	27.85	270.81
8	101.50	89.11	26.87	75.23	10.87	244.55
9	109.94	109.36	32.97	64.96	7.13	282.52
10	83.05	111.57	33.64	14.92	1.48	337.46
11	47.21	63.56	19.16	13.54	0.00	190.10
28	0.00	0.00	0.00	0.00	0.00	0.00

Results

Before parsley consumption, apigenin could not be detected in any plasma sample. Consumption of parsley led to a rise in apigenin plasma concentration in every subject (fig. 2). Maximum concentrations ranged from 27.85 to 337.46 nmol/l and were detected between 6 and 10 h after parsley consumption (table 1). However, the range of variation between subjects was high. Within 28 h, plasma concentrations fell under the detection limit (2.3 nmol/l) in each case. On the basis of the plasma apigenin concentrations, an average AUC (area under the curve) of 61.98 ± 48.56 min·µmol/l (mean ± SD) was calculated with a wide range of 8.37–149.99 min·µmol/l between participants (table 2). No effect of gender or sports activity

Table 2. Individual and summarized data on apigenin intake by parsley bolus, apigenin content in 24-hour urine nmol/l, area under the curve (AUC, min·µmol/l), maximum apigenin content in plasma nmol/l, and calculated apigenin excretion rate in urine (in % of the ingested amount of apigenin)

Subjects	Apigenin in parsley bolus nmol	Apigenin in 24 h urine nmol	AUC min·µmol/l	Maximum apigenin content in plasma nmol/l	Excretion rate in urine, % of ingested apigenin
1	87,995.75	261.80	86.64	236.13	0.30
2	74,796.39	243.20	70.32	307.80	0.33
3	73,034.99	89.71	11.10	80.06	0.12
4	45,757.79	167.47	58.05	270.81	0.37
5	51,037.53	26.46	31.94	134.42	0.05
6	51,037.53	46.73	45.95	194.27	0.09
7	63,356.94	31.37	11.49	53.25	0.05
8	52,797.45	59.71	149.99	227.81	0.11
9	56,317.28	274.00	8.37	27.85	0.49
10	79,196.17	310.52	138.93	337.46	0.39
11	87,995.75	70.98	69.05	244.55	0.08
Mean	65,756.69	143.81	61.98	192.22	0.22
SD	15,493.64	109.66	48.56	104.32	0.16
SEM	4,671.51	33.06	14.64	31.45	0.05
Min	45,757.79	26.46	8.37	27.85	0.05
Max	87,995.75	310.52	149.99	337.46	0.49

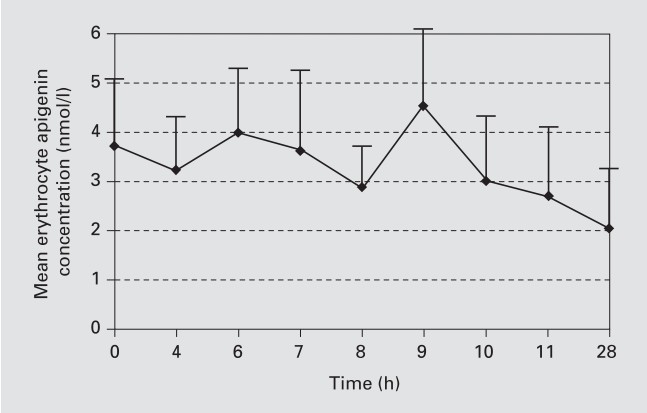

Fig. 3. Mean (± SEM) erythrocyte apigenin concentration (nmol/l) after parsley bolus.

on plasma apigenin concentration was found. No increase in querectin, isorhamnetin or luteolin plasma concentrations was noted after consumption of the parsley bolus.

Apigenin was detected in all 24-hour urine samples. Urine volumes ranged between 1.5 and 8.1 liters within 24 h with a mean of 3.7 ± 2.2 liters. On average, 0.039 ± 0.03 mg apigenin – corresponding to 143.81 ± 109.65 nmol/24 h – were detected in the 24-hour urine samples. As compared to the ingested apigenin dose of 17.77 ± 4.19 mg (65.76 ± 15.49 µmol), a mean excretion rate of 0.22 ± 0.16% was determined for apigenin (table 2).

Apigenin concentrations of 2.55–12.00 nmol/l could be detected in RBC samples of 8 participants (fig. 3); this is in many cases close to the detection limit (2.3 nmol/l). An enrichment of apigenin after parsley consumption as found in the plasma was not found. In 5 out of 8 subjects the RBC apigenin concentrations fell under the detection limit (2.3 nmol/l) within 28 h.

Discussion

The results of this study confirmed that after ingestion of an apigenin-rich food, apigenin is enriched in the human circulation. However, maximum plasma concentrations were comparably low (0.34 µmol/l) and on average only 0.22% of the ingested apigenin dose was found in the 24-hour urine samples.

It should be emphasized that we estimated the aglycon concentration of apigenin after acid (parsley) or enzymic (biological specimens) hydrolysis irrespective of the un-

derlying apigenin-containing compound. In food, apigenin is present almost exclusively as glycoside while in humans apigenin is subject to methylation, sulfation, and glucuronidation [3]. This should be kept in mind when discussing the present results.

Two previously published studies tried to determine apigenin bioavailability after consumption of parsley. In a randomized cross-over study with oral administration of 5 g dried parsley (containing 84 mg apigenin) for 7 days, apigenin could not be detected in plasma, most likely because the limit of detection of the applied methodology was relatively high with 1.1 μmol/l [11]. Thus, the authors assumed that apigenin absorption could generally be excluded, a statement which can be disapproved according to our findings. Nielsen et al. [12] detected apigenin in urine samples of 14 participants of a randomized cross-over trail after having administered a parsley supplement providing 3.73–4.49 mg apigenin/MJ. During parsley intervention, significantly more apigenin was excreted (20.7–5,727.3 μg/24 h) as compared to the apigenin-free baseline diet (0–1,571.7 μg/24 h; $p < 0.05$). The urinary excretion rate was estimated to be 0.58 ± 0.16% of ingested apigenin [12]. This is in good agreement with the findings of the present study (0.22 ± 0.16%). The authors suggested that the main part of the ingested apigenin was either excreted unabsorbed or was rapidly metabolized after absorption.

An active transport has been suggested for quercetin glucosides [3]. More likely, apigenin or apigenin-containing compounds are taken up by passive diffusion, probably also by means of membrane carriers. It seems that only aglycons and some polyphenol glucosides can be absorbed in the intestine whereas other glycosides can be hydrolyzed by the microflora in the colon. The low excretion rate of apigenin in our study may hint towards the last option. The study participants ingested 17.77 ± 4.19 mg apigenin which is a fairly high amount as compared to the estimated 'usual' intake of about 0.85 ± 0.87 mg/day in Hungarian adults [5]. It seems possible that such a high dose may have led to lower absorption rates through passive transportation than could have been achieved with smaller amounts.

Other flavonoids in parsley could have interacted with apigenin absorption; according to our results, parsley contained also appreciable amounts of quercetin, isorhamnetin and luteolin (myricetin not estimated). In addition, the apigenin content of parsley used in our study was rather low (11.89 mg/100 g edible portion) as compared to other data [11, 12, 15, 17], implying the need for a high amount of parsley to get the achieved apigenin dose. Therefore, interactions between flavonoids cannot be excluded. With respect to the plasma flavonoid concentrations, we did not find an increase in querectin, isorhamnetin or luteolin concentrations after the short-term intervention.

The participants of the present study excreted very different urine volumes (1.5–8.1 l/day). The remedy parsley which has been used for centuries is said to possess a strongly diuretic effect which was demonstrated in rats after consumption of parsley [18]. Parsley consumption leads to a decrease in Na^+/K^+-ATPase activity resulting in a reduction of the renal Na^+ and K^+ re-absorption and osmotic difference in renal cells and lumen (diuresis). It has not been examined yet which substance in parsley is responsible for such effects. Bearing the different urine volumes in mind, the participants probably reacted differently on the diuretic substances in parsley.

Concerning the formation of apigenin metabolites, Nielsen and Dragsted [19] tried to detect the suggested apigenin metabolite acacetin (4′-methoxyapigenin) in urine samples of humans after parsley consumption but failed. Using rat liver models, apigenin was shown to be converted into three monohydroxylated derivatives, luteolin (major metabolite), scutellarein and iso-scutellarein [10]. In our study, plasma luteolin concentrations showed no response to parsley consumption (scutellarein and iso-scutellarein could not be determined). With a four-site perfused rat intestinal model, flavonoid absorption was tested by Chen et al. [20]. When comparing flavones (apigenin) and isoflavones (genistein), the following results were obtained: (i) maximal amounts of intestinal conjugates excreted were 61 and 150 nmol/30 min for genistein and apigenin, respectively; apigenin conjugates were excreted much faster than genistein conjugates; (ii) apigenin absorption rate was highest in colon (40%) and lowest in ileum (21%); with the exception of the duodenum, more apigenin than genistein was absorbed, and (iii) in plasma, genistein conjugates were found at amounts at least 8 times higher than apigenin conjugates [20].

It seems worth mentioning that Nielsen et al. [12] were able to demonstrate a biological effect of parsley intervention on antioxidant enzyme activities. Both glutathione reductase and superoxide dismutase activities increased during the intervention phase. In the present study it could be shown that apigenin can be found in human RBC. This finding supports the hypothesis that certain flavonoids, including apigenin, may contribute to the cellular antioxidant defense system [21] or may reveal other biological effects as found in test systems (see Introduc-

tion). Fiorani et al. [22] found that quercetin is efficiently taken up by human RBC via passive diffusion resulting in an accumulation of the flavonoid in body cells. The substance was utilized as substrate for a trans-plasma membrane oxidoreductase contributing to the reduction of extracellular oxidants. Only in the presence of albumin was the substance released from RBC. Due to the structural similarity between quercetin and apigenin, these results can probably be transferred to the group of flavones as well. It has been suggested that human RBC could play a pivotal role in the distribution and bioavailability of circulating flavonoids [22].

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