

ORIGINAL COMMUNICATION

Absorption of kaempferol from endive, a source of kaempferol-3-glucuronide, in humans

MS DuPont¹, AJ Day², RN Bennett¹, FA Mellon¹ and PA Kroon^{1*}

¹Institute of Food Research, Norwich Research Park, Colney Lane, Norwich, UK; and ²Procter Department of Food Science, University of Leeds, Leeds, UK

Objective: To determine the absorption, excretion and metabolism of kaempferol in humans.

Design: A pharmacokinetic study of kaempferol from endive over 24 h.

Subjects: Four healthy males and four healthy females.

Results: Kaempferol, from a relatively low dose (9 mg), was absorbed from endive with a mean maximum plasma concentration of 0.1 µM, at a time of 5.8 h, indicating absorption from the distal section of the small intestine and/or the colon. Although a 7.5-fold interindividual variation between the highest and lowest maximum plasma concentration was observed, most individuals showed remarkably consistent pharmacokinetic profiles. This contrasts with profiles for other flavonoids that are absorbed predominantly from the large intestine (eg rutin). An average of 1.9% of the kaempferol dose was excreted in 24 h. Most subjects also showed an early absorption peak, probably corresponding to kaempferol-3-glucoside, present at a level of 14% in the endive. Kaempferol-3-glucuronide was the major compound detected in plasma and urine. Quercetin was not detected in plasma or urine indicating a lack of phase I hydroxylation of kaempferol.

Conclusions: Kaempferol is absorbed more efficiently than quercetin in humans even at low oral doses. The predominant form in plasma is a 3-glucuronide conjugate, and interindividual variation in absorption and excretion is low, suggesting that urinary kaempferol could be used as a biomarker for exposure.

Sponsorship: Biotechnology and Biological Sciences Research council for core strategic funding and the University of Leeds for a departmental fellowship (AJD).

European Journal of Clinical Nutrition (2004) 58, 947–954. doi:10.1038/sj.ejcn.1601916

Keywords: flavonol; kaempferol glucuronide; flavonoid; human absorption; metabolism; biomarker

Introduction

Flavonoids are biologically active polyphenolic compounds found in plants and present in plant-derived foods that are intrinsic components of human diets. They are widely distributed in the plant kingdom, formed as secondary metabolites through the phenylpropanoid biosynthetic pathway, but the levels and chemical forms vary markedly

depending on the plant source. Flavonoids have been shown to possess a range of biological activities that are consistent with them contributing to the protection afforded by a diet rich in fruit and vegetables against degenerative diseases such as cancer, diabetes, cardiovascular diseases and cataract (Block *et al*, 1992; Steinmetz & Potter, 1996; Knekt *et al*, 2002). Flavonoids are potent antioxidants *in vitro* and may function as such *in vivo* (Rice-Evans, 2001). They are also able to alter gene expression (eg induce expression of phase-II detoxification enzymes, or reduce expression of phase-I carcinogen-activating enzymes), inhibit the activity of radical generating enzymes (eg cyclooxygenases, lipoxygenase and xanthine oxidase), chelate iron, induce apoptosis, inhibit the proliferation of cancer cells, inhibit nitric oxide synthesis and reduce inflammation (see Birt *et al*, 2001; Nijveldt *et al*, 2001; Yang *et al*, 2001).

The ability of biologically active dietary flavonoids to influence cellular function and ultimately affect health

*Correspondence: PA Kroon, Nutrition Division, Institute of Food Research, Norwich Research Park, Norwich NR4 7UA, UK.

E-mail: paul.kroon@bbsrc.ac.uk

Guarantors: PA Kroon and AJ Day

Contributors: MSD and AJD designed the intervention; MSD supervised the intervention and prepared extracted samples; MSD and FM performed the chromatography and mass spectrometry; MSD, AJD, RB and FM interpreted the data; AJD and PAK wrote the manuscript. All contributors read and commented on the manuscript.

Received 9 May 2003; revised 20 August 2003; accepted 15 September 2003

usually requires absorption from the gastrointestinal tract and delivery to the peripheral blood in order to reach target tissues. A clear exception is the gastrointestinal tract itself, where the cells lining the tract can interact directly with nonabsorbed flavonoids or products of their microbial fermentation, or respond to changes in the composition of the microflora. Various approaches have been taken to assess the exposure of humans to flavonoids, including assessment of intake, determination of plasma kinetics following acute doses and measurements of (urinary) excretion. The intakes, plasma pharmacokinetics and urinary excretion of a few flavonoids and the soy isoflavones have been fairly extensively studied in man. For example, studies with quercetin have shown that various factors influence the amount absorbed, including the plant source and the type and linkage position of sugar(s) (Hollman *et al*, 1997, 1999; Erlund *et al*, 2000; Graefe *et al*, 2001). Further, recent studies have shown that quercetin (and other flavonoids and isoflavones) are present in plasma and urine as a mixture of conjugated forms (methylated/glucuronidated/sulphated) (Day *et al*, 2001; Clark *et al*, 2002). Therefore, in order to understand mechanisms and perform meaningful *in vitro* studies concerning potential health effects of flavonoids, it is critical to know how much is absorbed, what concentrations are relevant *in vivo* and the structures of the metabolites.

Flavonols, a subset of related structures within the flavonoids, are present in human diets predominantly as quercetin and kaempferol. Quercetin was shown to be the single biggest contributor to flavonol intake in consumption studies conducted in The Netherlands (Hertog *et al*, 1993) and the USA (Sampson *et al*, 2002). Nevertheless, kaempferol contributes significantly to flavonoid intake in humans (eg kaempferol accounted for 25–33% of mean total flavonol intake, with intakes estimated at 6–10 mg per day in the USA and the Netherlands (Hertog *et al*, 1993; Sampson *et al*, 2002). Whereas there are a relatively large number of studies concerning the absorption of quercetin, the bioavailability of kaempferol has been largely ignored. Kaempferol is structurally similar to quercetin and is expected to be biologically active. Furthermore, in a recent epidemiological study assessing individual flavonoid intake with chronic disease, high kaempferol intake was significantly correlated to a reduced risk of cerebrovascular disease (relative risk 0.7, $P=0.003$; Knekt *et al*, 2002). There are few data concerning kaempferol's absorption or excretion, and the structures of putative metabolites are not known.

Endive is a rich source of kaempferol, containing up to 246 mg kaempferol per kg fresh weight (DuPont *et al*, 2000). Further, the majority of the kaempferol in endive is present as an uronic acid conjugate (kaempferol-3-glucuronide) rather than a glycoside; nothing is known about the absorption of glucuronosyl flavonoid conjugates, which also enter the gut via the bile duct after metabolism in the liver (ie enterohepatic circulation). The aim of this study was to investigate the absorption of kaempferol in humans. We fed volunteers an endive soup and determined changes with

time in the total amount of kaempferol in plasma, the total amount excreted in urine and identified the major metabolites present in plasma and urine.

Materials and methods

Materials

Kaempferol standard was purchased from Extrasynthese (Genay, France), and the internal standard 3,4,5-trimethoxycinnamate from Avocado Research Chemicals Ltd (Lancaster, UK). β -Glucuronidase Type IX-A from *Escherichia Coli*, and sulphatase Type H-6 from *Helix pomatia* were purchased from Sigma (Poole, UK). All reagents were of analytical grade or HPLC grade where applicable. Water was purified via a Millex Q-plus system (Millipore, Watford, UK).

The endive soup test meal was prepared by the diet cook in the Human Nutrition Unit at the Institute of Food Research and contained curly endive (150 g raw content) cooked with potato (~100 g), bacon (~18 g), butter (~9 g) and water: each portion of soup had a final mass of 300 g and contained 150 g (precooked mass) of endive. Portions of soup (300 g) were individually frozen at -20°C until required, then thawed overnight and heated thoroughly by a microwave before serving.

Subjects

Eight healthy subjects (four females, four males) were recruited locally (from the Norwich Research Park) by advertisement: age ranged from 26 to 47 y, body mass index ranged from 21.5–31.3 kg/m². Prior to the study, a fasting blood sample from each subject was screened for biochemical suitability (fasting blood glucose, full blood count, urea and electrolytes, liver function tests and cholesterol) through the Norfolk and Norwich University Hospital Trust, and all were considered normal. Subjects were not taking medication. The study was approved by the Norwich District Research Ethics Committee and was performed under their guidelines.

Study protocol

Subjects were requested not to eat any flavonoid-rich foods for 48 h prior to, and during, the study; the list included, no fruits or juices, green or leafy vegetables, onions, tomatoes or beverages such as tea and red wine. The day before the study, subjects were asked to collect urine for 24 h and to fast from 22.00. On the study day, subjects arrived at the Human Nutrition Unit between 08.00 and 09.00 h. Immediately prior to eating the test meal, a sample of blood (25 ml) was taken as a baseline control ($t=0$) via a cannula. Subjects were then required to consume a bowl of thick endive soup (300 g) with a slice of white bread and a glass of water within an approximate time window of 10 min (range 7–15 min). After completion of the test meal, timing began for the study and blood samples (25 ml) were taken at 0.5, 1, 2, 3, 4, 6 and 8 h.

Subjects returned to the Unit the following morning for a 24 h blood sample to be taken. Subjects continued collection of urine throughout the study day (24 h). Water was offered to drink after 2 h as requested. A polyphenol-free meal was eaten at $t = 4$ h in the Unit, and after $t = 8.5$ h.

Plasma was prepared immediately from all samples by centrifugation in Rohre 10 ml lithium-heparin tubes (Sarstedt Ltd, Leicester, UK) at 1500 g for 10 min. The plasma was separated from the red blood cells and after addition of ascorbic acid (final concentration 1 mM), samples were frozen on dry ice. Samples were stored at -20°C until analysed. Urine samples were collected in bottles containing ascorbic acid (2 g) and frozen at -20°C until analysed.

Extraction of flavonols from plasma and urine

Plasma samples (10–15 ml) were incubated with phosphate buffer (3 ml, 0.1 M, pH 6.2), internal standard 3,4,5 trimethoxy cinnamate (100 μg), β -glucuronidase (200 U) and sulphatase (20 U) at 37°C for 3 h. Methanol (2 ml, containing 1 mM ascorbic acid), acetic acid (200 μl , 50%) and acetonitrile (to a final volume of 40 ml) were added to precipitate proteins and extract flavonols. The samples were vortex-mixed for 30 s every 2 min over a 10 min period, before centrifugation (13 600 g , 10 min, 4°C). The supernatant was evaporated to dryness, taken up in water/methanol (300–500 μl ; 1/1, v/v), vortex-mixed, microfuged (4 min, 8000 g), and passed through a 4 mm PVDF 0.2 μm syringe filter (Chromos Express, Macclesfield, UK) into vials for HPLC analysis.

Urine samples (20 ml) were incubated with phosphate buffer (3 ml, 0.1 M, pH 6.2), internal standard (100 μg), β -glucuronidase (200 U) and sulphatase (20 U) for 3 h at 37°C . Methanol and acetonitrile were added and samples were extracted as above. Evaporated samples were taken up in methanol/water (300 μl ; 1/1, v/v) and filtered for HPLC analysis.

All urine samples and plasma samples from selected subjects were also extracted directly into methanol/acetonitrile without prior hydrolysis. Extracted samples were then prepared as above for HPLC analysis.

HPLC analysis

A Hewlett-Packard 1100 system comprising a cooled autosampler, gradient mixer and a quaternary pump coupled to a diode array detector and controlled by Chemstation software was used. Solvents were A: water/tetrahydrofuran/trifluoroacetic acid (98/2/0.1, v/v/v) and B: acetonitrile, pumped at a flow rate of 1 ml/min. Samples (30 μl) were injected directly on to a Prodigy 5 μm ODS3 reversed-phase silica column (250 mm \times 4.6 mm i.d.) with guard (30 mm \times 4.6 mm i.d.) (Phenomenex Ltd., Macclesfield), held at a constant temperature (30°C). The effluent was monitored by diode array detection between 200 and 450 nm. The gradient system used was: 17% (solvent B) for 2 min, increasing to 25% at

7 min, 35% at 15 min, 48% at 20–23 min, 50% at 25 min, to 90% at 35 min and held 5 min before decreasing to 17% at 45 min, followed by post-run equilibrium for 10 min. An external kaempferol standard was run every six samples. The limit of detection for kaempferol in plasma was determined as 0.002 μM (the injected sample LOD was 0.51 ng = 2 pmol).

LC/MS analysis

Positive and negative ion electrospray LC/MS measurements were performed using a Micromass Quattro II (Manchester, UK) equipped with a Z-sprayTM source. Samples were introduced using a Hewlett-Packard 1050 HPLC equipped with a diode array detector. A solvent gradient as above was used for LC/MS. Eluent flow (1 ml/min) was split between the diode array detector and the mass spectrometer ion source in the approximate ratio 8:1. The Electrospray capillary voltage was set to 3.5 kV in positive ion mode and 2.5 kV in negative ion mode, and the cone voltage was set to 28 V. Source block temperature was 140°C and desolvation temperature 350°C . Nitrogen was used as the drying and nebulising gas at flows of 400 and 20 L/h, respectively.

Selected ion monitoring for plasma and urine samples was conducted on mass channels, positive mode: 239.09 (3,4,5-trimethoxycinnamic acid [Aglycone + H]⁺), 287.06 (kaempferol [Aglycone + H]⁺), 303.03 (quercetin [Aglycone + H]⁺), 309.04 (kaempferol [Aglycone + Na]⁺), 317.05 (Monomethyl-quercetin [Aglycone + H]⁺), 367.06 (kaempferol monosulphate [M + H]⁺), 389.04 (kaempferol monosulphate [M + Na]⁺), 463.13 (kaempferol monoglucuronide [M + H]⁺), 485.11 (kaempferol monoglucuronide [M + Na]⁺), 543.13 (kaempferol monoglucuronide monosulphate [M + H]⁺), 639.20 (kaempferol diglucuronide [M + H]⁺), 719.20 (kaempferol diglucuronide, monosulphate, [M + H]⁺); negative mode 237 (3,4,5-trimethoxycinnamic acid [Aglycone-H]⁻), 285.06 (kaempferol, [Aglycone-H]⁻), 365.06 (kaempferol monosulphate [M-H]⁻), 461.13 (kaempferol monoglucuronide [M-H]⁻), 541 (kaempferol monoglucuronide, monosulphate, [M-H]⁻), 637.20 (kaempferol diglucuronide [M-H]⁻), 717.20 (kaempferol diglucuronide, monosulphate [M-H]⁻), with a scan window of 0.2 μm , dwell times of 0.1 s/channel and an interchannel delay of cycle time of 0.03 s. The limit of detection of kaempferol in plasma was determined as 0.003 μM (the injected sample LOD was 0.75 ng = 3 pmol). Full scan spectra were obtained in negative mode from m/z 50–1000 at scan rate of 40/min and cone voltage 28. Concurrent diode array spectra were scanned from 200 to 450 nm, with an interval of 2 nm. Instrument control, data acquisition and processing were performed using Micromass MassLynxTM version 3.4 data system and software.

Results

Several batches of curly endive harvested during different growing seasons were analysed for their kaempferol-3-

glucuronide to kaempferol-3-glucoside ratio. Summer endive was used for the soup and contained 79% kaempferol-3-glucuronide, 14% kaempferol-3-glucoside and 7% kaempferol-3-(6-malonyl)-glucoside. Identification of peaks was based on previous work, where compounds had been fully characterised by NMR (DuPont *et al*, 2000). Quercetin was not detected in the endive samples. The soup portions provided 8.65 mg kaempferol equivalent. Freezing, thawing and microwave reheating of the soup did not alter the kaempferol content or profile.

The concentration of kaempferol in extracted plasma samples was checked after storage over 10 days. Kaempferol was found to be stable with or without the addition of acetic acid, which is in contrast to quercetin where an acid pH is required to prevent oxidative degradation (Day, 2000). Figure 1 shows the individual plasma pharmacokinetic profile of kaempferol (in enzyme hydrolysed samples) over the first 8 h for the eight subjects, with the inset showing the average plasma kaempferol concentrations over 24 h. Four subjects had nondetectable kaempferol plasma concentrations at 24 h (confirmed by LC/MS with a limit of detection of 0.003 μM). Plasma levels in other subjects returned to a concentration similar to 0.5 h after ingestion of the endive soup by 24 h. All but one subject showed early absorption of kaempferol, which appeared to peak after 0.9 h at $0.05 \pm 0.01 \mu\text{M}$. The maximum plasma concentration of kaempferol after 5.8 h was $0.10 \pm 0.01 \mu\text{M}$. One subject only exhibited detectable plasma levels of kaempferol at 4 h, and this was 7.5-fold lower (0.02 μM) than the highest individual (0.15 μM). The pharmacokinetic profile of the other seven subjects was remarkably consistent, and there were no

differences observed between the male and female subjects. The average per cent kaempferol excreted in 24 h was $1.9 \pm 0.3\%$ and was consistent for seven individuals, with one subject (the same as with low plasma kaempferol) excreting 10-fold less (0.3%) than the highest (2.9%). All the results are summarised in Table 1.

Plasma samples from four individuals at selected time points and urine samples from all subjects were analysed for kaempferol metabolites. Free kaempferol was detected in all plasma and urine samples, with 40% (s.d. ± 26) of total kaempferol in a free form in plasma, and 16% (s.d. ± 10) free in urine (Figure 2a). Quercetin and methylquercetin were not detected by selected ion monitoring LC/MS at m/z 303 and 317, respectively. Kaempferol-3-glucuronide was detected in unhydrolysed plasma and urine samples by LC/MS and confirmed by coelution with a pure standard and UV/visible spectra (Figure 2b). No other kaempferol

Table 1 Pharmacokinetic variables of kaempferol plasma absorption and urinary excretion in humans after ingestion of kaempferol-3-glucuronide from endive

Pharmacokinetic parameter	Value ^a
Plasma peak 1 concentration ($\mu\text{mol/l}$)	0.05 ± 0.01
Plasma peak 2 concentration ($\mu\text{mol/l}$)	0.10 ± 0.01
Time to reach peak 1 concentration (h)	0.9 ± 0.1
Time to reach peak 2 concentration (h)	5.8 ± 0.5
AUC _(0-24h) ($\text{h}/\mu\text{mol/l}$)	0.75 ± 0.15
Kaempferol excretion ($\mu\text{g}/24 \text{ h}$)	160 ± 24
Kaempferol excretion (%)	1.9 ± 0.3

^aValues are means \pm s.e., $n = 8$.

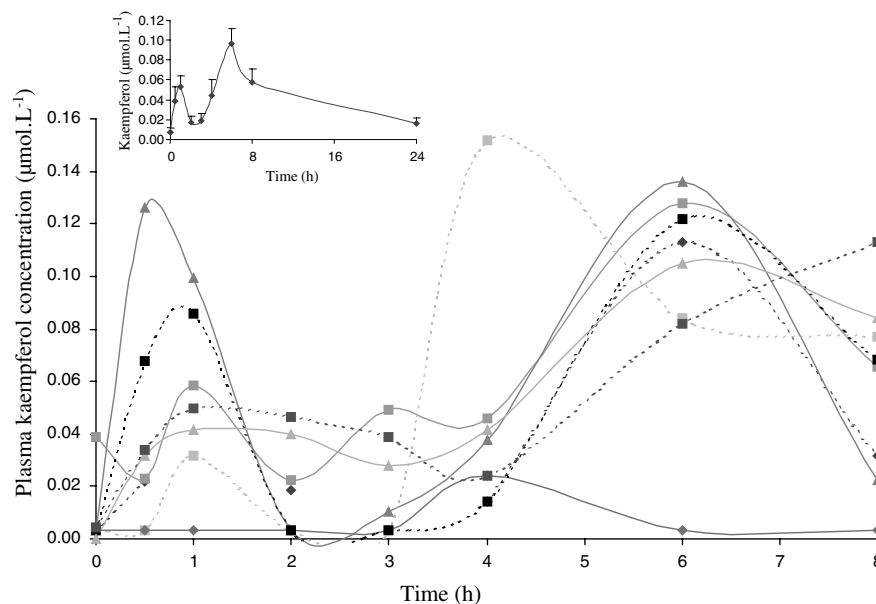


Figure 1 Plasma kaempferol concentration profile of individual subjects after consumption of kaempferol-3-glucuronide-rich endive. The data were obtained by analysing samples by HPLC with UV detection (wavelength 370 nm). Dotted lines indicate female subjects, solid lines indicate male subjects. Inset shows the average plasma concentration of kaempferol over 24 h; error bars are s.e., $n = 8$.

metabolites (eg mono- or di-sulphates, glucuronides) could be confirmed unequivocally by LC/MS in any of the plasma samples. In urine samples, there were two peaks that were putatively identified as a kaempferol monosulfate (based on retention time and UV/visible spectra (Day, 2000), and

LC/MS with SIM; m/z 365 and 285; Figure 2c) and a kaempferol disulphate (based on LC/MS SIM data only; m/z 445 and 285). However, the quality of the full scan MS spectra was insufficient to confirm identities.

Discussion

Only a few studies have investigated kaempferol absorption. De Vries *et al* (1998) measured the concentration of kaempferol and quercetin in the plasma and urine of individuals after a 7 day randomised crossover study following consumption of tea and onions. Kaempferol, only found in the tea, was excreted at 2.5% of the amount ingested compared with quercetin from tea that was excreted at a level of 0.5% of the amount ingested. These results suggest that either kaempferol is absorbed more efficiently than quercetin, that quercetin is preferentially excreted through the bile compared with kaempferol, or that quercetin is more efficiently converted to other compounds (in the gut, or postabsorption). In tea, kaempferol and quercetin are conjugated to similar moieties (mainly rutinosides; Price *et al*, 1998), but in onions, quercetin is found conjugated to glucosides (Price & Rhodes, 1997). Quercetin was excreted at an average of 1.1% of intake after onion consumption compared with 0.5% after tea, suggesting that quercetin from onions is more bioavailable than from tea. Recent studies have confirmed that the glycoside moiety is the major determinant of site of absorption of quercetin from the gastrointestinal tract, with quercetin rutinosides absorbed mainly from the colon and quercetin glucosides absorbed early in the small intestine (Morand *et al*, 2000; Olthof *et al*, 2000; Graefe *et al*, 2001).

Kaempferol from endive was excreted at 1.9% of the amount ingested, which is comparable with the levels in urine shown by De Vries *et al* (1998), as their samples were taken after 7 days of an intervention study. Furthermore, the concentration of kaempferol in plasma after 4 h in our study, 0.05 μM , was the same as in the study of De Vries (plasma samples were taken 4 h after ingestion of the first dose of tea, containing 9 mg kaempferol). In another study, Nielsen *et al* (1997) showed that kaempferol was excreted at 0.9% of the amount ingested from broccoli, although only two subjects were assessed.

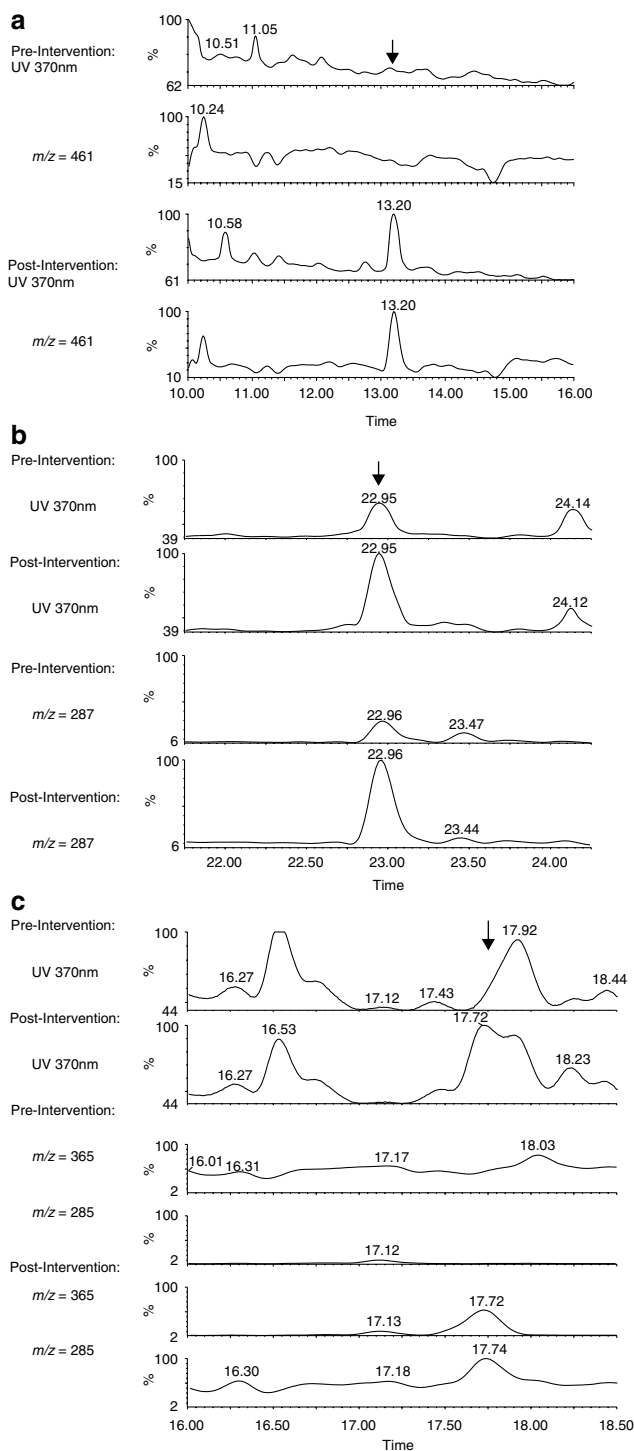


Figure 2 Identification of kaempferol and kaempferol metabolites in plasma and urine of human volunteers following ingestion of endive. The identities of metabolites were confirmed using a combination of retention time, UV spectra, full scan and selected ion monitoring LC/MS and comparison with pure authentic standards: (a) Kaempferol-3-O-glucuronide in plasma confirmed by a peak absorbance at 370 nm and presence of $m/z = 461$ ion using MS in selected ion monitoring mode (The m/z 285 fragment ion was not detected due to the reduced response (~ 5 -fold) of this ion, as demonstrated for the authentic standard); (b) Unconjugated kaempferol in plasma (UV 370 nm and m/z 287); (c) Kaempferol monosulfate in urine (UV 370 nm, m/z 365 and 285). The elution positions of authentic standards are indicated by the arrows.

Plasma and urinary kaempferol levels showed remarkable consistency between individuals (with the exception of one subject who showed very little absorption). The time of maximum absorption corresponds to absorption of kaempferol in the distal section of the small intestine (5.8 h), presumably due to the requirement for microflora to hydrolyse the β -glucuronide prior to uptake of the aglycone. In contrast, other flavonoid conjugates that require microbial metabolism prior to absorption across the colon, for example quercetin-3-rhamnoglucoside (rutin) and apigenin-7--apioside (apiin), show a much greater interindividual variation in absorption and excretion (Nielsen *et al*, 1999; Erlund *et al*, 2000). Differences in the bacterial population, affected by other dietary constituents (Rowland *et al*, 2000), may alter the rate and extent of conjugate hydrolysis and/or the rate of aglycone ring modification that may account for the large inter-individual variation observed in these studies. Thus, little interindividual variation in urinary kaempferol after endive consumption, released from the conjugate by microbial metabolism, suggests that urinary kaempferol could be a good biomarker for kaempferol consumption.

An early absorption peak of kaempferol in plasma was observed after consumption of endive soup. The most likely explanation is preferential absorption of kaempferol-3-glucoside present in the endive soup at 14% of total kaempferol. Quercetin glucosides are preferentially absorbed from the small intestine by mechanisms that almost certainly involve active transport (eg via SGLT1; Gee *et al*, 2000) and deglycosylation processes (eg lactase phlorizin hydrolase and cytosolic β -glucosidase; Day *et al*, 2000b, 2003; Nemeth *et al*, 2003). Kaempferol glucosides are almost certainly absorbed from the small intestine via similar processes due to the high degree of structural similarity between kaempferol glucosides and quercetin glucosides. Kaempferol-3-glucoside is a substrate for lactase phlorizin hydrolase, whereas kaempferol-3-(6-malonyl)-glucoside and kaempferol-3-glucuronide are poor substrates for lactase phlorizin hydrolase (Nemeth *et al*, 2003). It is unlikely that kaempferol-3-glucuronide is absorbed directly from the jejunum, as the compound is relatively hydrophilic and will be charged at the pH of the small intestine, thus reducing the ability of the compound to diffuse across the biological membrane. However, an active transport mechanism cannot be ruled out without further investigation. Furthermore, the time taken to reach the maximum plasma concentration in our study provides evidence for little absorption in the small intestine with the main absorption site being the distal small intestine and/or colon where microbial degradation will occur.

Nielsen *et al* (1997) detected kaempferol but not quercetin in urine after subjects consumed broccoli in a 12 day intervention, despite quercetin comprising 40% of the flavonol content of broccoli. These results suggest both that quercetin is more poorly absorbed than kaempferol, and that kaempferol is not converted to quercetin by phase I metabolism. We could also not detect quercetin in plasma or

urine after subjects consumed kaempferol-rich endive. Hydroxylation is an example of phase I cytochrome P450 mono-oxygenase-dependent activities that may be involved in the metabolism of flavonols. Nielsen *et al* (1998) showed that microsomes prepared from normal and Aroclor-1254-induced rats were capable of hydroxylating certain flavonols and flavones. The requirement for the metabolic activity was that either one or no hydroxyl groups were present on the B-ring. Two or more hydroxyl groups in the B-ring prevented further hydroxylation. These results have also been demonstrated in microsomes from human and mouse liver, and membrane isolates from *E. coli* expressing specific cytochrome P450 enzymes (Breinholt *et al*, 2002). However, when female rats were administered 100 mg of various flavonol and flavone aglycones, the 4'-hydroxylated flavonoids were recovered in the urine in an unchanged form (Nielsen, 1998). In human cell culture and human intervention studies, chrysin- and apigenin-hydroxylated metabolites were not detected (Galijatovic *et al*, 1999; Walle *et al*, 2001). These results suggest that hydroxylation of 4'-hydroxyflavonoids is not a common metabolic route *in vivo*.

Once absorbed, kaempferol will undergo phase II metabolism; conjugation with glucuronide or sulphate are the most likely metabolic routes and have been shown for quercetin (Day *et al*, 2001). Conjugation is a common detoxification reaction leading to increased solubility of compounds and a higher molecular weight, which is important for excretion particularly in the bile. As some quercetin metabolites found *in vivo* retain biological activities assessed *in vitro* (Day *et al*, 2000a), it is important to identify kaempferol *in vivo* metabolites in order to assess the potential biological activity of the actual circulating forms of this compound. In plasma, we could only unequivocally identify one conjugated kaempferol metabolite, namely kaempferol-3-glucuronide, which accounted for 55–80% of total kaempferol (>95% of the kaempferol conjugates). Although it is possible that the circulating compound results from absorption of the original glucuronide in endive, it is more likely that the compound was deconjugated by gut microflora β -glucuronidase in the colon prior to absorption. This would explain the high T_{max} for plasma total kaempferol. The aglycone was then reconstituted by UDP-glucuronosyltransferase in the small intestine or liver (Day *et al*, 2000a; Oliveira & Watson, 2000). Quercetin-3-glucuronide retained the ability of quercetin to inhibit LDL oxidation (Moon *et al*, 2001; Terao *et al*, 2001), but further work is required to determine the effect of conjugation on bioactivity of kaempferol-3-glucuronide. A single kaempferol mono-sulphate was tentatively identified by selected ion monitoring LC/MS, which, based on evidence from metabolism of kaempferol by human hepatocytes (Day, 2000), is likely to be kaempferol-7-sulphate. The concentration of the metabolites was too low to obtain a confirmatory full scan. We found no evidence of kaempferol-7-glucuronide in any samples (lack of UV absorbance at a retention time equivalent to that of the authentic standard). Although there is some evidence to suggest that glucuroni-

dation of flavonols at the 7-position does not occur *in vivo* (no quercetin-7-O-glucuronide was present in plasma of volunteers fed onions; Day *et al*, 2001), human liver cell-free extracts and microsomal preparations generate the 7-glucuronide of quercetin as the major product (Day *et al*, 2000a; Boersma *et al*, 2002), and human hepatocytes produce kaempferol-7-glucuronide when treated with kaempferol (Day, 2000). The cause of these differences (*in vivo* compared with *in vitro/ex vivo*) are not clear, but may in part be due to differences in accessibility between cell-free systems and intact cells. A high level of nonconjugated kaempferol was detected in the plasma and urine. This was somewhat surprising since no unconjugated quercetin was detected in plasma or urine of volunteers fed considerable amounts of quercetin from onions (Day *et al*, 2001). However, it is possible that the unconjugated kaempferol observed in this study resulted from endogenous β -glucuronidase activity. Kaempferol-3-glucuronide was shown to be a substrate for human recombinant β -glucuronidase (O'Leary *et al*, 2001). The catalytic efficiency of the enzyme was highest for kaempferol-3-glucuronide compared with the other flavonoid glucuronides tested, which may explain why unconjugated quercetin has not been found in plasma.

To the best of our knowledge, this is the first work to describe the pharmacokinetic absorption of a flavonoid from an orally dosed glucuronide conjugate. Kaempferol-3-glucuronide was absorbed from endive soup at a time corresponding with transit through the latter part of the small intestine. Microbial metabolism appeared necessary for the hydrolysis of the β -glucuronide conjugate, but consistent plasma concentration profiles and per cent excreted in the urine over 24 h suggest that urinary kaempferol could be used as a biomarker for kaempferol absorption. The plasma concentrations of metabolites after consumption of 9 mg of kaempferol are lower than the majority of biological activities so far shown *in vitro*. However, the greater urinary recovery of kaempferol compared with quercetin suggests an ability to increase plasma kaempferol significantly with increased consumption of kaempferol-rich foods, such as broccoli, kale, green beans, leeks and tea. Further work is required to characterise the kaempferol metabolites and assess their potential biological activity at realistic plasma concentrations.

Acknowledgements

We thank all the volunteers, the staff of the Human Nutrition Unit, especially Yvonne Clements for preparation of endive soup and the nurses Aliceon Blair, Linda Oram and Lesley Maloney for looking after the subjects, and John Eagles for additional mass spectrometric measurements.

References

Birt DF, Hendrich S & Wang WQ (2001): Dietary agents in cancer prevention: flavonoids and isoflavonoids. *Pharmacol. Ther.* **90**, 157–177.

- Block G, Patterson B & Subar A (1992): Fruit, vegetables and cancer prevention: a review of the epidemiological evidence. *Nutr. Cancer* **18**, 1–29.
- Boersma MG, van der Woude H, Bogaards J, Boeren S, Vervoort J, Cnubben NH, van Iersel ML, van Bladeren PJ & Rietjens IM (2002): Regioselectivity of phase II metabolism of luteolin and quercetin by UDP-glucuronosyl transferases. *Chem. Res. Toxicol.* **15**, 662–670.
- Breinholt VM, Offord EA, Brouwer C, Nielsen SE, Brosen K & Friedberg T (2002): *In vitro* investigation of cytochrome P450-mediated metabolism of dietary flavonoids. *Food Chem. Toxicol.* **40**, 609–616.
- Clark DB, Lloyd A, Botting N, Oldfield M, Needs P & Wiseman H (2002): Measurement of intact sulphate and glucuronide phytoestrogen conjugates in human urine using isotope dilution liquid chromatography–tandem mass spectrometry with [¹³C(3)]isoflavone internal standards. *Anal Biochem* **309**, 158–172.
- Day AJ (2000): Human absorption and metabolism of flavonoid glycosides. PhD dissertation, University of East Anglia, UK.
- Day AJ, Cañada FJ, Díaz JC, Kroon PA, Mclauchlan R, Faulds CB, Plumb GW, Morgan MRA & Williamson G (2000b): Dietary flavonoid and isoflavone glycosides are hydrolysed by the lactase site of lactase phlorizin hydrolase. *FEBS Lett.* **468**, 166–170.
- Day AJ, Bao Y-P, Morgan MRA & Williamson G (2000a): Conjugation position of quercetin glucuronides and effect on biological activity. *Free Radical. Biol. Med.* **29**, 1234–1243.
- Day AJ, Mellon F, Barron D, Sarrazin G, Morgan MRA & Williamson G (2001): Human metabolism of dietary flavonoids: identification of plasma metabolites of quercetin. *Free Rad. Res.* **35**, 941–952.
- Day AJ, Gee JM, DuPont MS, Johnson IT & Williamson G (2003): Absorption of quercetin-3-glucoside and quercetin-4'-glucoside in the rat small intestine: the role of lactase phlorizin hydrolase and the sodium-dependent glucose transporter. *Biochem. Pharmacol.* **65**, 1199–1206.
- DeVries JHM, Hollman PCH, Meyboom S, Buysman MNCP, Zock PL, van Staveren WA & Katan MB (1998): Plasma concentrations and urinary excretion of the antioxidant flavonols quercetin and kaempferol as biomarkers for dietary intake. *Am. J. Clin. Nutr.* **68**, 60–65.
- DuPont MS, Mondin Z, Williamson G & Price KR (2000): Effect of variety, processing, and storage on the flavonoid glycoside content and composition of lettuce and endive. *J. Agri. Food Chem.* **48**, 3957–3964.
- Erlund I, Kosonen T, Alftan G, Maenpaa J, Perttunen K, Kenraali J, Parantainen J & Aro A (2000): Pharmacokinetics of quercetin from quercetin aglycone and rutin in healthy volunteers. *Eur. J. Clin. Pharmacol.* **56**, 545–553.
- Galijatovic A, Otake Y, Walle UK & Walle T (1999): Extensive metabolism of the flavonoid chrysin by human Caco-2 and Hep G2 cells. *Xenobiotica* **29**, 1241–1256.
- Gee JM, DuPont MS, Day AJ, Plumb GW, Williamson G & Johnson IT (2000): Intestinal transport of quercetin glycosides in rats involves both deglycosylation and interaction with the hexose transport pathway. *J. Nutr.* **130**, 2765–2771.
- Graefe EU, Wittig J, Mueller S, Riethling AK, Uehleke B, Drewelow B, Pforte H, Jacobasch G, Derendorf H & Veit M (2001): Pharmacokinetics and bioavailability of quercetin glycosides in humans. *J. Clin. Pharmacol.* **41**, 492–499.
- Hertog MGL, Hollman PCH, Katan MB & Kromhout D (1993): Intake of potentially anticarcinogenic flavonoids and their determinants in adults in The Netherlands. *Nutr. Cancer* **20**, 21–29.
- Hollman PCH, Buysman MNCP, van Gameren Y, Cnossen EPJ, deVries JHM & Katan MB (1999): The sugar moiety is a major determinant of the absorption of dietary flavonoid glycosides in man. *Free Rad. Res.* **31**, 569–573.
- Hollman PCH, van Trijp JMP, Buysman MNCP, Gaag MS, Menglers MJB, deVries JHM & Katan MB (1997): Relative bioavailability of the antioxidant quercetin from various foods in man. *FEBS Lett.* **418**, 152–156.
- Knekt P, Kumpulainen J, Jarvinen R, Rissanen H, Heliovaara M, Reunanen A, Hakulinen T & Aromaa A (2002): Flavonoid intake and risk of chronic diseases. *Am. J. Clin. Nutr.* **76**, 560–568.

- Moon JH, Tsushida T, Nakahara K & Terao J (2001): Identification of quercetin 3-O-beta-D-glucuronide as an antioxidative metabolite in rat plasma after oral administration of quercetin. *Free Rad. Biol. Med.* **30**, 1274–1285.
- Morand C, Manach C, Crespy V & Remesy C (2000): Quercetin 3-O-beta-glucoside is better absorbed than other quercetin forms and is not present in rat plasma. *Free Rad. Res.* **33**, 667–672.
- Nemeth K, Plumb GW, Berrin JG, Juge N, Jacob R, Naim HY, Williamson G, Swallow DM & Kroon PA (2003): Deglycosylation by small intestinal epithelial cell beta-glucosidases is a critical step in the absorption and metabolism of dietary flavonoid glycosides in humans. *Eur. J. Nutr.* **42**, 29–42.
- Nielsen SE (1998): Metabolism and biomarker studies of dietary flavonoids. PhD Thesis, Danish Veterinary and Food Administration, Denmark.
- Nielsen SE, Breinholt V, Justesen U, Cornett C & Dragsted LO (1998): *In vitro* biotransformation of flavonoids by rat liver microsomes. *Xenobiotica* **28**, 389–401.
- Nielsen SE, Kall M, Justesen U, Schou A & Dragsted LO (1997): Human absorption and excretion of flavonoids after broccoli consumption. *Cancer Lett.* **114**, 173–174.
- Nielsen SE, Young JF, Daneshvar B, Lauridsen ST, Knuthsen P, Sandstrom B & Dragsted LO (1999): Effect of parsley (*Petroselinium crispum*) intake on urinary apigenin excretion, blood antioxidant enzymes and biomarkers for oxidative stress in human subjects. *Br. J. Nutr.* **81**, 447–455.
- Nijveldt RJ, van Nood E, van Hoorn DEC, Boelens PG, van Norren K & van Leeuwen PAM (2001): Flavonoids: a review of probable mechanisms of action and potential applications. *Am. J. Clin. Nutr.* **74**, 418–425.
- O'Leary KA, Day AJ, Needs PW, Sly WS, O'Brien NM & Williamson G (2001): Flavonoid glucuronides are substrates for human liver beta-glucuronidase. *FEBS Lett.* **503**, 103–106.
- Oliveira EJ & Watson DG (2000): *In vitro* glucuronidation of kaempferol and quercetin by human UGT-1A9 microsomes. *FEBS Lett.* **471**, 1–6.
- Olthof MR, Hollman PCH, Vree TB & Katan MB (2000): Bioavailabilities of quercetin-3-glucoside and quercetin-4'-glucoside do not differ in humans. *J. Nutr.* **130**, 1200–1203.
- Price KR & Rhodes MJC (1997): Analysis of the major flavonol glycosides present in four varieties of onion (*Allium cepa*) and changes in composition resulting from autolysis. *J. Sci. Food Agric.* **74**, 331–339.
- Price KR, Rhodes MJC & Barnes KA (1998): Flavonol glycoside content and composition of tea infusions made from commercially available teas and tea products. *J. Agric. Food Chem.* **46**, 2517–2522.
- Rice-Evans C (2001): Flavonoid antioxidants. *Curr. Med. Chem.* **8**, 797–807.
- Rowland IR, Wiseman H, Sanders TAB, Adlercreutz H & Bowey EA (2000): Interindividual variation in metabolism of soy isoflavones and lignans: influence of habitual diet on equal production by the gut microflora. *Nutr. Cancer* **36**, 27–32.
- Sampson L, Rimm E, Hollman PCH, de Vries JHM & Katan MB (2002): Flavonol and flavone intakes in US health professionals. *J. Am. Diet. Assoc.* **102**, 1414–1420.
- Steinmetz KA & Potter JD (1996): Vegetables, fruit, and cancer prevention: a review. *J. Am. Diet. Assoc.* **96**, 1027–1039.
- Terao J, Yamaguchi S, Shirai M, Miyoshi M, Moon JH, Oshima S, Inakuma T, Tsushida T & Kato Y (2001): Protection by quercetin and quercetin 3-O-beta-glucuronide of peroxynitrite-induced antioxidant consumption in human plasma low-density lipoprotein. *Free Rad. Res.* **35**, 925–931.
- Walle T, Otake Y, Brubaker JA, Walle UK & Halushka PV (2001): Disposition and metabolism of the flavonoid chrysin in normal volunteers. *Br. J. Clin. Pharmacol.* **51**, 143–146.
- Yang CS, Landau JM, Huang MT & Newmark HL (2001): Inhibition of carcinogenesis by dietary polyphenolic compounds. *Ann. Rev. Nutr.* **21**, 381–406.