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Absorption of Ferulic Acid from Low-Alcohol Beer

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Flavonoids and monophenolic compounds have been well-described over recent years for their properties as antioxidants and scavengers of reactive oxygen and nitrogen species. A number of epidemiological studies implicate a role for flavonoids in reducing the risk of coronary heart disease. In particular, the focus has been on flavonol-rich fruit and vegetables and flavonoid-rich beverages, especially tea and red wine. Mechanisms of protection are unclear since the absorption, distribution, metabolism and elimination of dietary phenolics have not yet been extensively investigated. Here we report the bioavailability of ferulic acid, 4-hydroxy-3-methoxy-cinnamic acid, the major hydroxycinnamate in beer. Studies of the pharmacokinetics of urinary excretion of ferulic acid from low alcohol beer consumption in humans have been undertaken. The results show that ferulic acid is absorbed with a peak time for maximal excretion of *ca.* 8 h and the mean cumulative amount excreted is 5.8 ± 3.2 mg. These findings are consistent with the uptake of ferulic acid from dietary sources, such as tomatoes, and suggest that ferulic acid is more bioavailable than individual dietary flavonoids and phenolics so far studied.

Keywords: Phenolic absorption, ferulic acid, beer, HPLC

INTRODUCTION

There is considerable evidence for a role for foods rich in antioxidant nutrients, vitamin E, vitamin C and β-carotene, in the maintenance of health and reduction in risk of cancer and cardiovascular diseases. Recent work is also beginning to highlight a potential role for flavonoid and phenolic components of fruit, vegetables, beverages and grains.[1–16]

Flavonoids and phenolic secondary metabolites of plants are effective antioxidants *in vitro*, through their H-donating properties, and are capable of scavenging reactive oxygen species (reviewed in [17]) and reactive nitrogen species.[18–21] The total antioxidant potential of phenolic-rich fruit, vegetables and beverages[22–25] has been assessed in studies which have utilised assays of total antioxidant activity. Beverages such as beers, white wine and apple juice demonstrate antioxidant activities of the same order, but much less than those of red wines.[25]

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Consumption of red wine and tea has been associated with health benefits due to their flavonoid content, although little is actually known about the absorption, distribution, metabolism and excretion of the phenolic components. Recent human studies have identified quercetin glycosides, dihydrochalcone glycosides and anthocyanins in plasma.[26–28] Conjugates and metabolites of phenolics have also been detected in urine as a result of the consumption of pure compounds, dietary components and plant extracts. Most of the bioavailability studies have focused on the flavonol glycosides, quercetin and kaempferol, the flavanone glycosides naringin and hesperidin, the catechins, the anthocyanins and the hydroxycinnamates.[29–39]

The major raw materials of beer are malted barley and hops, both of which are rich in flavonoids.[40,41] Ferulic acid is a constituent of barley cell walls, while hops contain predominantly condensed polyphenolic materials. It is to be expected, therefore, that beer could be a relatively rich source of flavonoids.[40,41]

The purpose of this study was to investigate the uptake in humans of the hydroxycinnamate ferulic acid from beer as measured by urinary excretion. The results show that ferulic acid is a major bioavailable phenolic from beer and that its absorption profile is similar to that from a fruit source such as fresh tomato.

MATERIALS AND METHODS

Human Study and Supplementation

Ethical permission was obtained from Guy's Hospital Research Ethics Committee. Five healthy males, mean age 28 years (range 23–40), with mean BMI 25 kg/m^2 (range 21–28) were recruited to the study. The volunteers were resident in the Guy's Drug Research Unit for two days and maintained on a low flavonoid diet for one day prior to, and for the duration of, the study period. The diet specifically avoided all fresh fruit, vegetables, wholegrain and wheat products (wholemeal bread, wholemeal cereals, crispbreads, breakfast cereal, biscuits, porridge, cakes and pastries), specific beverages (fruit juices, wines, coffee, cola drinks and other beers) and other products such as jams, jellies, chocolate and tomato ketchup.

Due to the design of the study a low alcohol beer was applied to conform to the requirements of the Hospital Research Ethics Committee concerning alcohol ingestion and the time period over which it was to be consumed. A beer was brewed by Brewing Research International specifically for this project. The malt was a commercial ale type. The hop variety chosen was the commercial variety Fuggles. This variety is often used for traditional British "real ales" and is relatively low in bittering potential. This means that relatively high concentrations of hops are used per litre of beer brewed, thus maximising the contribution of hop polyphenols to the beer. The alcohol content of the beer was low (approximately 1% alcohol by volume) in order to minimise the alcohol intake of the volunteers. The mass of malt used per litre of beer produced was 0.147 kg (1.5 kg crystal malt + 13.5 kg ale malt/102 l). The mass of hops used was 2.48 g/l beer (253 g/102 l). Volunteers consumed 7 pints (4 l) over 4 h.

Volunteers were screened and dietary restrictions introduced on day one. After 24 h, basal urine samples were collected and the beer consumption initiated immediately post low-flavonoid breakfast (Figure 1). All volunteers consumed each beer portion over approximately a 30 min time period. Urine samples were collected for 24 h from the initiation of beer consumption. Individual urine samples were collected in sterile tubes and stored at −70°C until analysis. Recorded adverse effects (due to

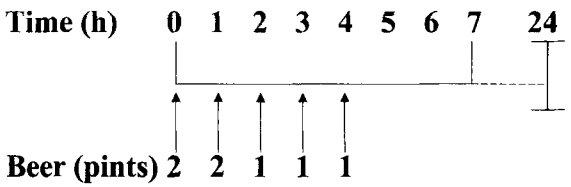

FIGURE 1 Supplementation schedule and urine collection period.

the palatability of the low alcohol, relatively sweet beer) were nausea and vomiting (in the case of volunteers MPG and MJH).

Chemicals

Methanol and acetonitrile, all HPLC grade were obtained from Rathburn Chemicals (Walkerburn, Scotland). Ferulic acid and all other hydroxycinnamates were obtained from Extra-synthese (ZI Lyon Nord, BP 62, 69730 Genay, France). Elgastat "ultra high pure" double-distilled water (18 Ω grade) was used in all experiments. β-Glucosidase was obtained from ICN Biomedicals Inc. (Ohio 44202, USA) and salicylic acid and β-glucuronidase (Type IX-A from E-Coli) was from Sigma (UK).

Stock solutions of the HPLC standards were prepared by dissolving 1–2 mg of sample into either methanol or mobile phase (20% (v/v) methanol, in 0.1% (w/v) HCl). Urine standards were prepared for analysis by the addition of ferulic acid stock solution to pooled human urine from the basal samples provided by the volunteers involved in the study. The standards ranged from 0 to 300 ng of ferulic acid. Salicylic acid was used as an internal standard for the HPLC analysis.

HPLC Analysis of the Phenolic Composition of Beer

A 20 ml volume of beer with 50 µl added internal standard (1 mg/ml salicylic acid) was extracted three times with 30 ml ethyl acetate. The combined extracts were passed through a filter containing anhydrous potassium carbonate, and dried down by rotary evaporation at 40–45°C. The phenolic extract was reconstituted in either 2 ml water or in 50 : 50 (v/v) methanol/water. Both extracts on analysis gave identical recoveries of the hydroxycinnamates of interest; however, extraction of less hydrophilic components, such naringenin, required the methanol/water system. To release phenolics from glycosides, 500 µl of the methanol-free extract was treated with 1 mg β-glucosidase (3811 U/mg) and the sample incubated at 37°C for 1 h. The sample was diluted with an equal volume of mobile phase prior to HPLC analysis.

HPLC analysis was carried out on a Hewlett Packard 1100 system equipped with Peltier cooled autosampler, quaternary pump with degasser, thermostated column compartment, diode array detector and software. A Nova-Pak C18 column (4.6 × 250 mm) with a 4 µm particle size was used and the temperature maintained by the column oven set at 30°C. The injection was by means of an autosampler and the volume injected was 30 µl. Elution (0.5 ml/min) was performed using a gradient solvent system comprising solvent A (water : methanol : 5 M HCl = 79.9 : 20 : 0.1) and solvent B, acetonitrile. The mobile phase consisted of 95% solvent A for 10 min and then decreased linearly to 50% solvent A at 50 min, back to 95% solvent A at 55 min and held at these conditions for a further 5 min. There was a 10 min delay before the next injection to ensure re-equilibration of the column. Authentic standard solutions were analysed as precision controls by being randomly placed with the samples as unknowns. The monitoring wavelength for the chromatograms was detected at 320 nm. Peak identification of each component was by retention time, from spectral characteristics of each peak by photodiode array detection from 200–600 nm, and confirmation by spiking with identified compounds.

Analysis of Urinary Phenolics by Gradient HPLC

Urine samples were thawed and mixed well. Samples were prepared for HPLC analysis as described previously.[36] A 1 ml sample was diluted into a 5 ml disposable culture tube containing 5 µl salicylic acid solution as internal standard (stock solution 2 mg/ml). To this, 2.4 ml of methanol and 100 µl of HCl (5 M) were added and the sample stoppered and mixed for 30 s. Samples were centrifuged at 800*g* for 10 min at 4°C, the supernatant collected, and methanol removed under nitrogen. The resultant aqueous fraction was filtered using a Flowpore 0.22 µm

sterile nonpyrogenic membrane filter (Whatman, UK) directly into an HPLC vial.

To cleave the glucuronidated conjugates, samples were hydrolysed with β-glucuronidase. For enzymatic hydrolysis 1 ml of urine containing 5 µl salicylic acid solution was incubated with 500 U/ml (final concentration) β-glucuronidase in a stoppered culture tube for 24 h at 37°C. Following incubation, 100 µl of HCl (5 M) and 2.4 ml methanol were added. The samples were stoppered and mixed for 30 s, as described above.

HPLC analysis was conducted according to the method of Paganga and Rice-Evans[28] using the Waters Millennium system. The HPLC system consisted of an autosampler with a Peltier temperature controller, a 626 pump with a 600S controller, a photodiode array detector, and a software system that controlled all the equipment and carried out data processing. HPLC analysis was carried out under the same conditions as the beer analysis except that the elution rate was 0.8 ml/min.

Quantification was carried out using calibration of the ferulic acid standard. Four calibration runs with ferulic acid were executed routinely. For the urine assay, calibration was performed by following the procedures for the standard solutions as described for urine samples. A linear regression calculation was performed on the resulting plot of peak area versus amount of ferulic acid, and the regression line used to calculate the amount of ferulic acid present.

RESULTS

Analysis of beer samples showed levels of 2.36 ± 0.15 mg/l ($n = 5$) free ferulic acid and 1.13 ± 0.06 mg/l *p*-coumaric acid ($n = 5$) (Figure 2) which did not change after treatment with β-glucosidase to cleave putative glycosides. The chromatogram suggests elution of ferulic acid at $R_t = 25.1$ min, confirmed by spectral characterisation and spiking with the hydroxycinnamate.

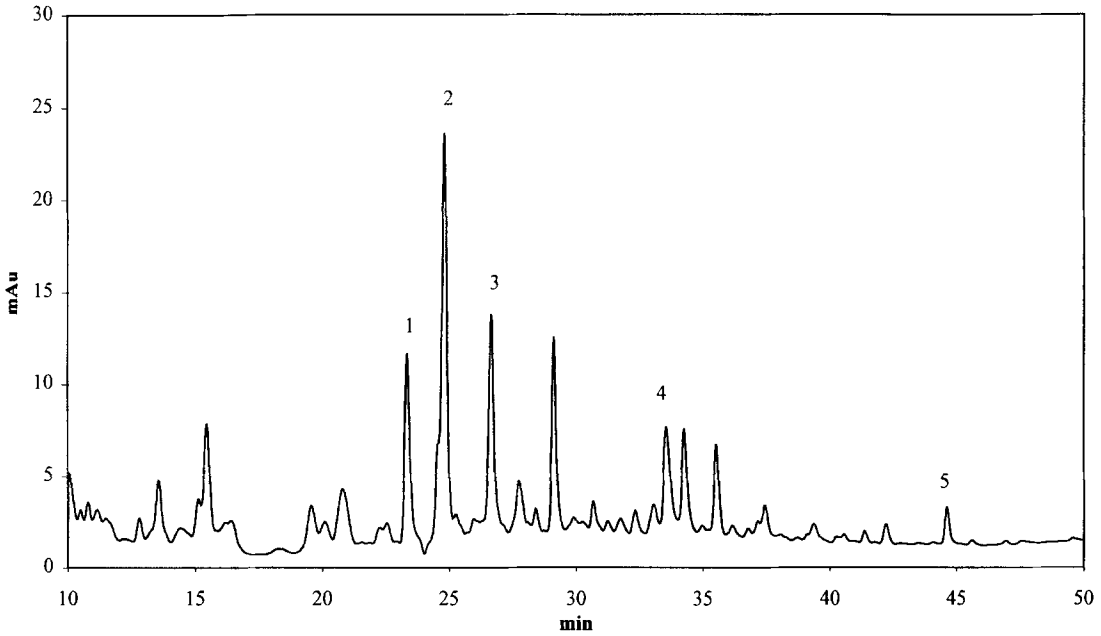

FIGURE 2 HPLC chromatogram of methanolic extract of beer depicting peaks 1–5 corresponding to 1 *p*-coumaric acid, 2 sinapic/ferulic acid, 3 flavonol, 4 salicylic acid (internal standard), 5 naringenin. The chromatographic conditions were as described in Materials and Methods section.

However, co-elution with a minor component is also suggested by the HPLC profile of the urine from some volunteers; spectral indexing identifies the component co-eluting with ferulic acid as sinapic acid. (5-methoxy ferulic acid), another hydroxycinnamic acid component of beer, having similar retention time and polarity. This is consistent with the observations of McMurrough *et al.*[41]

Cumulative excretion of total ferulic acid after ingestion of beer (Figure 3) increased with time post-supplementation giving a broadly similar profile for most of the volunteers. Excretion continued progressively for 8–10 h after which it reached a plateau, with no further excretion. The results presented are the combined values of free ferulic acid plus conjugated ferulic acid, as the glucuronide, quantified from the increase in ferulic acid detected by HPLC after treatment of the urine samples with β-glucuronidase. The relative proportions of the free and conjugated forms excreted varied from subject to subject, with a mean of $44 \pm 13\%$ of the total being in the conjugated form (Table I). The total amount of ferulic acid excreted after beer ingestion increased to a range of approximately 2–9 mg (mean 5.8 ± 3.2 mg). The total volume of urine excreted after 24 h ranged from 2.5 to 4 l. Figure 4 demonstrates the individual concentrations in the urine excreted at specific times for 2 representative subjects. On the concentration versus time plot, the time at which maximal excretion occurred was *ca.* 7–9 h (Figure 4).

TABLE I Relationship of urinary excretion of free and conjugated ferulic acid to total amount

Subject	Total ferulic acid	Glucuronide % of total	Free ferulic acid (mg)
MPG	1.8	39	1.1
MUT	5.5	62	2.1
MJH	3.9	36	2.5
PDD	9.3	30	6.6
SJP	8.7	54	4.0
		Mean 44 ± 13.3	

DISCUSSION

A range of epidemiological studies suggests the potential importance of the flavonoid non-nutrient components of the diet and their putative contribution to health benefit,[1–10] although some of the findings are conflicting.[11–16] An inverse association between intake of flavonoids and risk

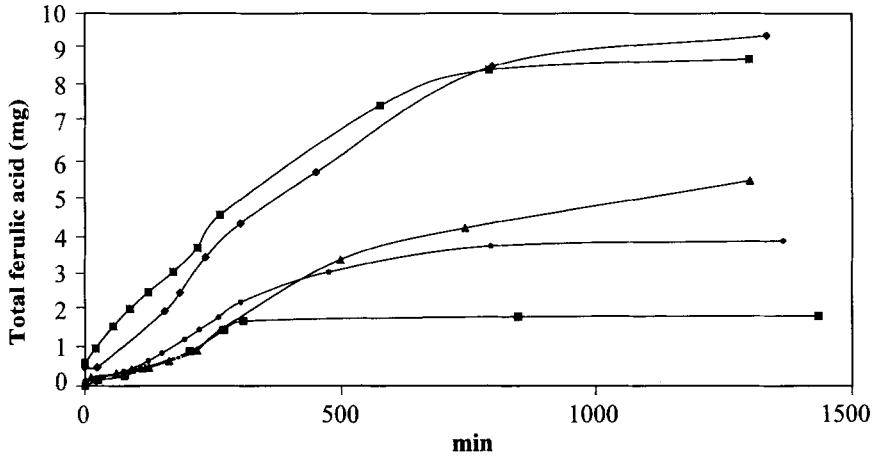

FIGURE 3 Cumulative urinary excretion over 24 h of total ferulic acid (free plus that recovered from feruloyl glucuronide) as a function of time for each subject (total amounts from subjects depicted by ● and ■ were lower due to adverse effects).

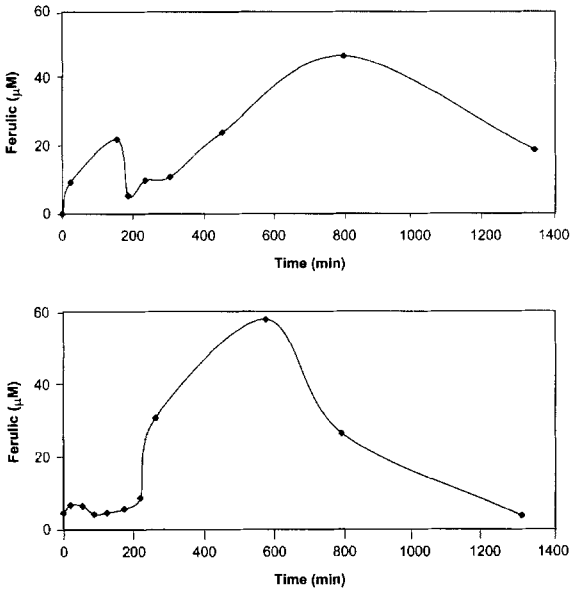

FIGURE 4 Absolute concentrations of ferulic acid excreted in urine as a function of time for two volunteers.

of coronary heart disease was found in both the Zutphen prospective cohort study[2] and the Seven Countries cross-cultural study.[3] Tea and flavonol intake were very strongly inversely associated with the risk of stroke.[4] Flavonol intake, especially derived from onions and apples, was also inversely correlated with coronary heart disease in Finnish populations.[5] More recently, studies have demonstrated a decreased risk of myocardial infarction with increased tea ingestion.[6] In a smaller study green tea polyphenols decreased platelet aggregation *ex vivo*.[10] The low incidence of coronary heart disease in the French, who consume a high fat diet, has been claimed to be attributed in part, to regular consumption of red wine.[1]

Others have shown that red wine intake increases the resistance of LDL to oxidation *ex vivo*[7,8] although this contrasts with an observed negative effect of alcohol-free wine consumption on this biomarker.[13] Similar contrasting findings concerning tea and LDL oxidation have been revealed.[9,14] Other major studies do not reveal an association between intake and cardiovascular disease: the Caerphilly study[11] demonstrated increased risk of death from coronary heart disease and tea consumption; the Health Professionals Study also found no association of flavonol intake with cardiovascular disease mortality or non-fatal myocardial infarction.[12] Protective effects of flavonols against cancer have been proposed[42] but epidemiological data fail to support this.[3,16] This begs the question as to why associations with flavonols were the major connections explored when there are other major flavonoid components in both teas and red wine. Hop flavonoids have been found to be inhibitory to the proliferation of human breast as ovarian cancer cell lines[43] as well as being inhibitory to the metabolic activation of carcinogens.[44]

These findings indicate that it is crucial to understand the absorption, distribution, metabolism and excretion of phenolics, their nature and metabolites and to discern the bioactive components and their mechanisms. The major reported phenolic components of beer are monophenols, especially the hydroxycinnamic acids *p*-coumaric, ferulic, chlorogenic and caffeic acids (including esters and glycosides), along with phenolic acids such as gallic acid, constituting 10–30 mg/l of beers overall, compared with the flavon-3-ols (1.5–23 mg/l), the anthocyanogens such as leucocyanidin (4–80 mg/l), the lesser constituent flavonols as glycosides of kaempferol, myricetin, isoquercitrin and rutin (< 10 mg/l), and the condensed polymerised catechins (20–60 mg/l).[40] Ferulic acid is the most abundant hydroxycinnamic acid in beer, reports finding ranges of 0.52, 1.13, 0.90 mg/l in ale, lager and stout, respectively, to 1.1, 2.0, 1.2 mg/l in Irish brewed ales, lagers and stouts.[41,45] Of these components the condensed polyphenols are too large to be absorbed in their native form and are likely to be cleaved prior to absorption. The monomeric flavon-3-ols are known from other studies to be extensively metabolised,[46,47] thus the major detectable components are likely to be the hydroxycinnamic acids.

The study described here provides evidence for the absorption and bioavailability of the hydroxycinnamate ferulic acid from beer

consumption. As the study design involved ingestion of 7 pints in 4 h, a low alcohol beer was specially brewed to satisfy the requirements of the Guy's Research Ethical Committee concerning alcohol intake over this time period. The peak time for maximal excretion is approximately 8 h and the recovery of ferulic acid in the urine, on the basis of the total amount of free ferulic acid and feruloyl glucuronide excreted, is greater than that of free ferulic acid and feruloyl glycosides ingested. However, beer also contains polymers of ferulic acid bound to cellulose as well as associated to proteins since phenolic acids are bound in the cellular walls of germinated barley but maybe extracted into wort during mashing. Thus both free and bound forms would be found in beer.[48,49] These findings closely match those detected on the uptake of ferulic acid from tomatoes – with maximal excretion at 7 h and the recovery of ferulic acid in the urine being 11–25% of that ingested.[36]

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