RESEARCH ARTICLE

Absorption of dimethoxycinnamic acid derivatives in vitro and pharmacokinetic profile in human plasma following coffee consumption

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Scope: This study reports the 24 h human plasma pharmacokinetics of 3,4-dimethoxycinnamic acid (dimethoxycinnamic acid) after consumption of coffee, and the membrane transport characteristics of certain dimethoxycinnamic acid derivatives, as present in coffee.
Methods and results: Eight healthy human volunteers consumed a low-polyphenol diet for 24 h before drinking 400 mL of commercially available coffee. Plasma samples were collected over 24 h and analyzed by HPLC-MS^2. Investigation of the mechanism of absorption and metabolism was performed using an intestinal Caco-2 cell model. For the first time, we show that dimethoxycinnamic acid appears in plasma as the free aglycone. The time to reach the C_{max} value of approximately 0.5 μM was rapid, $T_{max} = 30$ min, and showed an additional peak at 2–4 h for several subjects. In contrast, smaller amounts of dimethoxy-dihydrocinnamic acid ($C_{max} \sim 0.1$ μM) peaked between 8 and 12 h after coffee intake. In the cell model, dimethoxycinnamic acid was preferentially transported in the free form by passive diffusion, and a small amount of dimethoxycinnamoylquinic acid hydrolysis was observed.
Conclusion: These findings show that dimethoxycinnamic acid, previously identified in plasma after coffee consumption, was rapidly absorbed in the free form most likely by passive diffusion in the upper gastrointestinal tract.

Keywords:
Bioavailability / Caco-2 cells / Coffee / Human plasma / Phenolic acids

1 Introduction

Coffee is a rich source of chlorogenic acids (CGAs) consisting of a diverse family of esters formed between quinic acid and certain hydroxycinnamic acids (HCAs) such as caffeic acid, ferulic acid, and *p*-coumaric acid [1], which may be acylated at the C3, C4, and C5 position on the quinic acid. Dimethoxycinnamoylquinic acids (DQAs) and caffeoyl/feruloyl-dimethoxycinnamoylquinic acids di-esters (DCiQAs) have been identified previously [2] and the content is estimated at 0.15% on a dry mass basis (dmb), compared to 5% dmb for 5-*O*-caffeoylquinic acid (CQA). Recently, the occurrence of 3,4-dimethoxycinnamic acid (dimethoxycinnamic acid) was reported in Robusta coffee beans at a content of 0.02% dmb [3].

Epidemiological research indicates a beneficial association of coffee intake and reduced risk of type 2 diabetes [4] and colorectal cancer [5, 6]. A recent human trial demonstrated that regular coffee consumption led to increased serum levels of CGA metabolites and a significant lowering of biomarkers of inflammation and oxidative stress [7]. There are several proposed mechanisms that may explain the beneficial effects of coffee, such as improved •NO availability [8] and attenuation of NF-κB activation [9] by 5-*O*-caffeoylquinic acid.

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Abbreviations: **CGAs**, chlorogenic acids; **C_{max}**, maximum plasma concentration; **CQA**, caffeoylquinic acid; **DCiQAs**, caffeoyl/feruloyl-dimethoxycinnamoylquinic acids; **diDQA**, di-*O*-dimethoxycinnamoylquinic acid; **DQAs**, dimethoxycinnamoylquinic acids; **HHSS**, Hank's balanced Salt Solution; **HCAs**, hydroxycinnamic acids; **LLOQ**, lower limit of quantification; **MRM**, multiple reaction monitoring; **P_{app}**, permeability coefficient; **R_t**, retention time; **TEER**, transepithelial electrical resistance; **T_{max}**, time taken to reach plasma C_{max}

There is growing evidence that CGA metabolites may contribute to improved intestinal health by increasing mucosal membrane integrity via upregulating the expression of tight junction protein [10]. Additionally, favorable antiglycemic effects and modulation of oxidative enzymes have been observed in animal models of type 2 diabetes following consumption of caffeic acid [11] and ferulic acid [12]. It is now widely appreciated that chronic low-grade inflammation plays a key role in development of degenerative diseases [13], and the reported bioactivity of phenolic acids may in part contribute to their control or prevention.

Recently, dimethoxycinnamic acid was identified at high abundance in human plasma, after enzymatic treatment, 1 h after coffee consumption [14] despite the low concentration of dimethoxycinnamic acid or its derivatives in the original coffee. Dimethoxycinnamic acid is of interest because it is unlikely to be conjugated during first pass metabolism due to the absence of free hydroxyl groups on the C6 ring. The physicochemistry of this compound suggests that it can readily permeate across tissue barriers and may have improved or modified bioactivity. Further investigation is required to elucidate the form of metabolites present in the body after coffee intake and pharmacokinetic profile in healthy humans.

The results of this study provide new information concerning absorption of dimethoxycinnamic acid derivatives from instant coffee and show the interindividual variation of in vivo bioavailability over a 24-h period after coffee consumption by healthy volunteers.

2 Materials and methods

2.1 Chemicals and materials

All chemicals used in this investigation were purchased from Sigma-Aldrich (Berkshire, UK) unless stated otherwise. ACN (LC-MS grade), ethanol (99% purity), and nylon filters of pore size 0.2 μm, 0.4-μm diameter were purchased from Fisher Scientific Ltd. (Leicestershire, UK). All water refers to deionized Millipore water (Millipore UK Ltd., Hertfordshire, UK). Culture flasks, Transwell plates fitted with polycarbonate semipermeable inserts of pore size 0.4 μm and area 1.12 cm^2 or 4.67 cm^2 were obtained from Corning Life Sciences (Appleton Woods, Birmingham, UK). Chlorogenate esterase (Kikkoman, Japan) was kindly donated by Nestlé Research Center, Switzerland. 3,5-di-*O*-(3,4-) dimethoxycinnamoylquinic acid (3,5-diDQA) was kindly donated by the late Dr. Nigel Botting, University of St. Andrews, UK.

2.2 Cell culture

The human colon adenocarcinoma cell line, Caco-2 (HTB-37), was obtained from American Type Culture Collection at passage 18 (LGC Promochem, Middlesex, UK). Transport experiments utilized Caco-2 cells between passages 35 and 45. Caco-2 cells were seeded on Transwell inserts at a density 6×10^4 cells/cm^2 and cultured for 21 days at 37°C under a humidified 5% CO_2 atmosphere. DMEM culture medium was supplemented with 10% FBS, 584 mg/L L-glutamine, 100 U/mL penicillin-streptomycin, 1% (v/v) minimum essential medium, and 0.25 μg/mL amphotericin B and the medium was replaced every other day.

2.3 Transport and metabolism studies

The coffee solution used to investigate Caco-2 transport of dimethoxycinnamic acid derivatives was "Nescafé original decaffeinated" instant coffee. In brief, coffee granules (9, 18, and 27 mg/mL) were prepared in HBSS, modified with 1.8 mM calcium chloride; a sufficient volume was adjusted to pH 7.4 with NaOH (10 M) and centrifuged at 17 000 × *g* for 5 min to remove particulate matter. The resulting supernatants of three differing strengths were used as a source of dimethoxycinnamic acid and DQA derivatives and formed the apical test solutions in the described cell experiments.

On or after 22 days, time-course permeation studies were initiated by replacement of DMEM culture medium with HBSS modified with 1.8 mM calcium chloride (pH 7.4) in the apical (0.5 mL) and basal (1.5 mL) compartments. Plates were incubated at 37°C in a humidified 5% CO_2 atmosphere for 15 min to allow equilibration of tight junction integrity. Apical and basal solutions were carefully aspirated and apical test solution (18 mg/mL, pH 7.4) was added to the apical compartment, all basal solutions were HBSS (pH 7.4) modified with 1.8 mM calcium chloride. Trans-epithelial electrical resistance (TEER) was recorded using a Millicell ERS volt-ohm meter fitted with a chopstick probe (Millipore Ltd, Watford, UK) and the pH of the compartment solutions was measured (Twin pH meter B-212, Horiba Scientific, Middlesex, UK). A volume of the apical transport solution was added to a Transwell plate, in the absence of cells, as a reference sample of coffee composition and both the Transwell plate and reference sample were incubated at 37°C in a humidified 5% CO_2 atmosphere. Permeation was monitored by removal of a volume (100 μL) of modified HBSS from the basal compartment at 10, 20, 30, and 40 min, after each time sample the volume was replaced using fresh modified HBSS. At 40 min, TEER and pH measurements were repeated and then an aliquot of the apical (300 μL) and basal (900 μL) solution was collected, acetic acid was added to obtain a final concentration of 10 mM, and samples stored at –80°C. Permeation was performed in triplicate and is expressed as mean amount detected ± SD.

Concentration-dependent transport of dimethoxycinnamic acid was evaluated as described above using freshly prepared coffee solution at three different strengths, 9, 18, or 27 mg/mL. The relevant concentration was added (0.5 mL) to the apical compartments ($n = 3$ per concentration) and all basal solutions were HBSS (pH 7.4) modified with 1.8 mM calcium chloride. Control wells, without coffee solution, were prepared using HBSS (pH 7.4) modified with 1.8 mM calcium

chloride in both compartments ($n = 3$). At 30 min, TEER and pH measurements were recorded and samples stored as described above.

To investigate the permeation of 3,5-diDQA, the standard was dissolved at a high concentration in DMSO, diluted with modified HBSS to a final concentration of 500 μM, 0.2% DMSO, and transported as described using 6-well plates with 2-mL apical and basal chamber volumes.

The mean final monolayer resistance for each condition was corrected for cell-free membrane resistance and compared to the control to validate the monolayer integrity. The final TEER value for the control was 435 ± 52 Ωcm^2 and no statistical difference was observed between the control and all coffee solution test conditions ($p > 0.149$). For cells grown on polycarbonate inserts for 23 days, a TEER value of approximately 400 Ωcm^2 is indicative of a monolayer with established tight junctions and a well-developed apical-brush border [15].

Instant coffee samples and culture solutions were defrosted and deproteinated. In brief, each apical or basal solution (46 μL) was combined with 6.4 μL of 50% aqueous formic acid, ascorbic acid (final concentration 1 mM), and sinapic acid (final concentration 6 μM) as an internal standard. To the mixture, 112.5 μL of ACN was added dropwise to precipitate proteins and the samples were vortexed for 1 min and allowed to stand for 1 min, this was repeated three times and then samples were centrifuged at 17 000 × *g* for 5 min. The supernatant (100 μL) was removed and dried under centrifugal evaporation (Genevac EZ-2 plus, Suffolk, UK).

Cell metabolism was investigated by comparing the amount of dimethoxycinnamic acid detected in the apical and basal chambers to the amount detected in the absence of cells by HPLC-MS^2 analysis in multiple reaction monitoring (MRM) mode as described in Section 2.7.

2.4 Human study

The study design has been published in detail previously [14] and was approved by the ethical committee of clinical research of the University of Lausanne, Switzerland (Protocol reference 136/07). In brief, nine healthy volunteers (four males, five females) aged 27–41, weighting 70 ± 10 kg, and measuring 170 ± 8 cm in height were recruited, and in total eight subjects successfully completed the study. Volunteers avoided consumption of alcohol, coffee, cola, tea, wholegrain cereal, and medication for 24 h before the study was initiated. On the morning of the study, volunteers arrived in a fasted state and baseline blood samples were collected. Each subject then consumed 400 mL of commercially available instant coffee (10 mg/mL) and blood was collected at 0.5, 1, 1.5, 2, 3, 4, 5, 6, 8, 10, 11, 12, and 24 h after receiving the beverage. Standard polyphenols-free lunch and dinner were provided and water was available ad libitum. Blood was centrifuged for 10 min at 3500 × *g* (4°C) and 1 mL plasma aliquots were stored into cryotubes. Storage solution (40 μL) consisting of ascorbic acid (200 mg/mL) and EDTA (1 mg/mL) dissolved in 0.4 M sodium phosphate buffer (pH 3.6) was added to each cryotube. Samples were stored at –80°C prior to analysis.

2.5 Plasma extraction procedure

Samples corresponding to the maximum plasma concentration (C_{max}) of dimethoxycinnamic acid, measured after esterase hydrolysis, were selected for analysis. An aliquot of plasma for each subject was defrosted and submitted to extraction in the absence or presence of chlorogenate esterase (0.1 U) to assess the native form of dimethoxycinnamic acid in circulating fluids. The extraction technique was modified based on a previously described method [16]. In brief, 380 μL of plasma and 20 μL of 3-(4-hydroxyphenyl)-propionic acid as internal standard to a final amount of 500 nM (dissolved in 5% ACN in water (5:95, v/v) and 0.1% formic acid) were combined with 1200 μL of ethanol. The mixture was vortexed (2 min), left to stand (2 min) to allow protein to precipitate, and then centrifuged at 17 000 × *g* for 5 min. The ethanol supernatant was removed to a fresh 2-mL tube. The protein pellet was precipitated twice more using 400 μL of ethanol each time and the ethanol supernatants were pooled in the 2-mL tube. Combined ethanol supernatants were placed in a heated block (37°C) and dried under nitrogen flow (approximately 3 h). For samples intended for enzymatic reaction, the chlorogenate esterase (0.1 U) was dissolved in 400 μL of freshly prepared 50 mM sodium phosphate buffer (pH 6.5) and the enzyme mixture was added to the dried residue. Samples that were not treated with esterase were resuspended in 400 μL of 50 mM sodium phosphate buffer (pH 6.5) in the absence of enzyme. Samples were incubated (37°C) in a water bath for 1 h. At the end of the incubation, 42 μL of HCl was added to stop the enzymatic reaction and samples were vortexed briefly to mix. Immediately after, samples were submitted to liquid–liquid extraction. Ethyl acetate (500 μL) was added to the samples, vortexed to mix (4 min), and centrifuged at 17 000 × *g* for 5 min to separate the aqueous and ethyl acetate phases. The upper ethyl acetate volume was carefully removed to a fresh 2-mL tube. The extraction procedure was repeated four times and the ethyl acetate volumes were pooled in the 2-mL tube. Samples were placed in a heated (37°C) block and dried under nitrogen (approximately 45 min). The dried residue was immediately resuspended in 20 μL of warm ACN vortexed to mix (1 min), and then 80 μL of water was added and mixed for 2 min. Samples were centrifuged at 17 000 × *g* for 5 min and the supernatants were filtered using nylon membrane syringe filters. The filtrates were sealed in amber vials and stored under refrigeration (4°C) during HPLC-MS^2 analysis.

2.6 Pharmacokinetic analysis of dimethoxycinnamic acid derivatives in plasma

Extraction and enzymatic deconjugation of metabolites from plasma has been previously described [14] and is comparable

to the method presented in the current study. Immediately before analysis, samples were dissolved in 200 μL water: ACN (70:30, v/v) containing 1% acetic acid. A volume (10 μL) was injected onto an LC-MS system (Waters Acquity Ultra High Performance Liquid Chromatography (UPLC)) coupled with a Waters Synapt High Definition Mass Spectrometer) and full details of the operating conditions have been reported elsewhere [14]. The amounts of dimethoxycinnamic acid and 3,4-dimethoxy-dihydrocinnamic acid in plasma were quantified relative to ferulic acid, as no standard was commercially available, using matrix-match calibration curves. Maximum concentration of analyte in plasma from 0 to 24 h postingestion was defined as C_{max}, and T_{max} was the time taken to reach the maximal concentration.

2.7 Coffee, culture solution, and C_{max} plasma HPLC-MS^2 analysis

Analysis of the coffee, cell culture solutions, and extracted plasma samples for the C_{max} time point was performed using the following HPLC-DAD-MS^2 methodology. Briefly, a volume (5 μL) of sample was injected on to a Rapid Resolution HPLC-DAD (1200 series Agilent Technologies, Berkshire, UK). Chromatographic separation was achieved on an Eclipse plus C18 column (30 °C, 2.1 mm × 100 mm, 1.8 μm; Agilent Technologies) using a 68-min gradient of (A) premixed 5% ACN in water (5:95, v/v) and (B) premixed 5% water in ACN (5:95, v/v) both modified with 0.1% formic acid with a flow rate of 0.26 mL/min. Elution was initiated at 0% of solvent B and maintained for 17 min; the percentage of solvent B was then increased to 25% over the next 34 min and immediately increased to 100% for 5 min before initial starting conditions were resumed for a 12-min column reequilibration. MS operating conditions were optimized for dimethoxycinnamic acid, using the available standard, to obtaining a specific fragmentor voltage and collision energy. Standards were not available for 3-*O*-dimethoxycinnamoyl quinic acid (3-*O*-DQA), 4-*O*-dimethoxycinnamoyl quinic acid (4-*O*-DQA), and 5-*O*-dimethoxycinnamoyl quinic acid (5-*O*-DQA). Operating conditions for DQAs were obtained by postcolumn infusion of coffee solution from the LC to the ESI probe in negative mode using SIM mode to identify the optimum fragmentor voltage for transmittance to the mass analyzer. Similarly, a range of collision energies was assessed for each DQA in MRM mode and the condition that produced the highest abundance of distinct product ions for each compound was selected. DQAs and dimethoxycinnamic acid were detected using MRM mode based on retention time (R_t) of the dimethoxycinnamic acid standard or distinct MS^2 fragment patterns [2, 17], respectively, and quantified based on the major MS^2 product ion (base peak) using a calibration curve for dimethoxycinnamic acid. In the absence of standards, it was necessary to determine the analytical response of DQAs relative to dimethoxycinnamic acid. A crude correction factor for DQA quantification was calculated. In brief, instant coffee granules (18 mg/mL) were dissolved in hot water and vortexed to mix. A volume (20 μL) was added to 380 μL of water and triplicate samples were submitted to the plasma extraction procedure in the presence or absence of chlorogenate esterase (0.1 U) as described in Section 2.5. Based on the change in peak area after complete esterase hydrolysis of 5-*O*-DQA and conversion to dimethoxycinnamic acid, the response of 5-*O*-DQA was determined to be 20 times higher than that of dimethoxycinnamic acid and this factor was used to correct the quantification of all DQAs. Recovery experiments were performed for dimethoxycinnamic acid and used as a correction factor in the quantification of DQAs and dimethoxycinnamic acid.

HPLC-MS^2 methodology was validated as described in previous works [18] according to ICH recommendations [19] and the European Commission for the performance of analytical methods [20] for the identification and quantification of dimethoxycinnamic acid in plasma. Performance of the HPLC-MS was calculated based on dimethoxycinnamic acid at three concentrations; 150, 300, and 600 nM. Intra- and interday precisions were less than 4 and 8%, respectively. Intra- and interday accuracies were less than 10%. The LOQ and LOD were determined as 150 and 50 nM, respectively. Calibration curves showed good linearity ($R^2 = 0.99$) and this was confirmed by a Pearson's coefficient of 0.99 significant at the 1% level. Precision (RSD) and accuracy (RE) were within acceptable limits of ±15% [20].

2.8 Recovery experiments

Recovery was determined for the deproteination procedure, i.e., dimethoxycinnamic acid in either coffee transport solution (pH 7.4) or HBSS basal solution (pH 7.4), and the plasma extraction procedure. The recoveries were used as a correction factor for the quantification of dimethoxycinnamic

Table 1. Quantities of chlorogenic acids in the coffees used in this investigation

Chlorogenic acids	Percentage of dry weight basis	
	Human study	Caco-2 cell study
3-*O*-CQA	1.02	1.28
4-*O*-CQA	1.38	1.18
5-*O*-CQA	4	2.06
4-*O*-FQA	0.18	0.22
5-*O*-FQA	0.75	0.53
4,5-*O*-diCQA	0.54	0.11
3,4-*O*-diCQA	0.50	0.19
Total%	8.37	5.57

Values are expressed on a percentage of dry weight basis (g/100g) of coffee solids. The composition of the coffees was qualitatively the same, but absolute amounts showed some variations between the two samples.

acid and DQAs in the cell transport studies and the human plasma. In brief, a small volume of a high concentration of dimethoxycinnamic acid (250 mM) dissolved in DMSO was used to spike coffee solution (9 mg/mL) or HBSS (pH 7.4) at a final concentration of 25 or 10 μM. A volume (2 mL) was incubated in the absence of cells ($n = 5$) for 2 h at 37°C in a 5% CO_2 humidified atmosphere. Immediately after an aliquot (100 μL) was removed and submitted to the cell culture deproteination procedure as described in Sections 2.3. Dried samples were reconstituted (50 μL) in ACN in water (5:95, v/v), quantified by HPLC-MS^2 with authentic standards, and normalized on sinapic acid as the internal standard. For the coffee solution, the same stock was processed as described without spiking to determine the native quantity of dimethoxycinnamic acid. Recovery was determined as the amount detected in the spike (after correction for the native content in the coffee solution) and values are expressed as a percentage of the initial spike amount ± SD. The typical recovery for dimethoxycinnamic acid in coffee transport solution (pH 7.4) was 54 ± 1%, and in HBSS basal solution (pH 7.4) was 86 ± 4%.

For the extraction efficiency in plasma, dimethoxycinnamic acid was prepared at a high concentration in DMSO (10 mM) and diluted in ACN and water (5:95, v/v). A volume

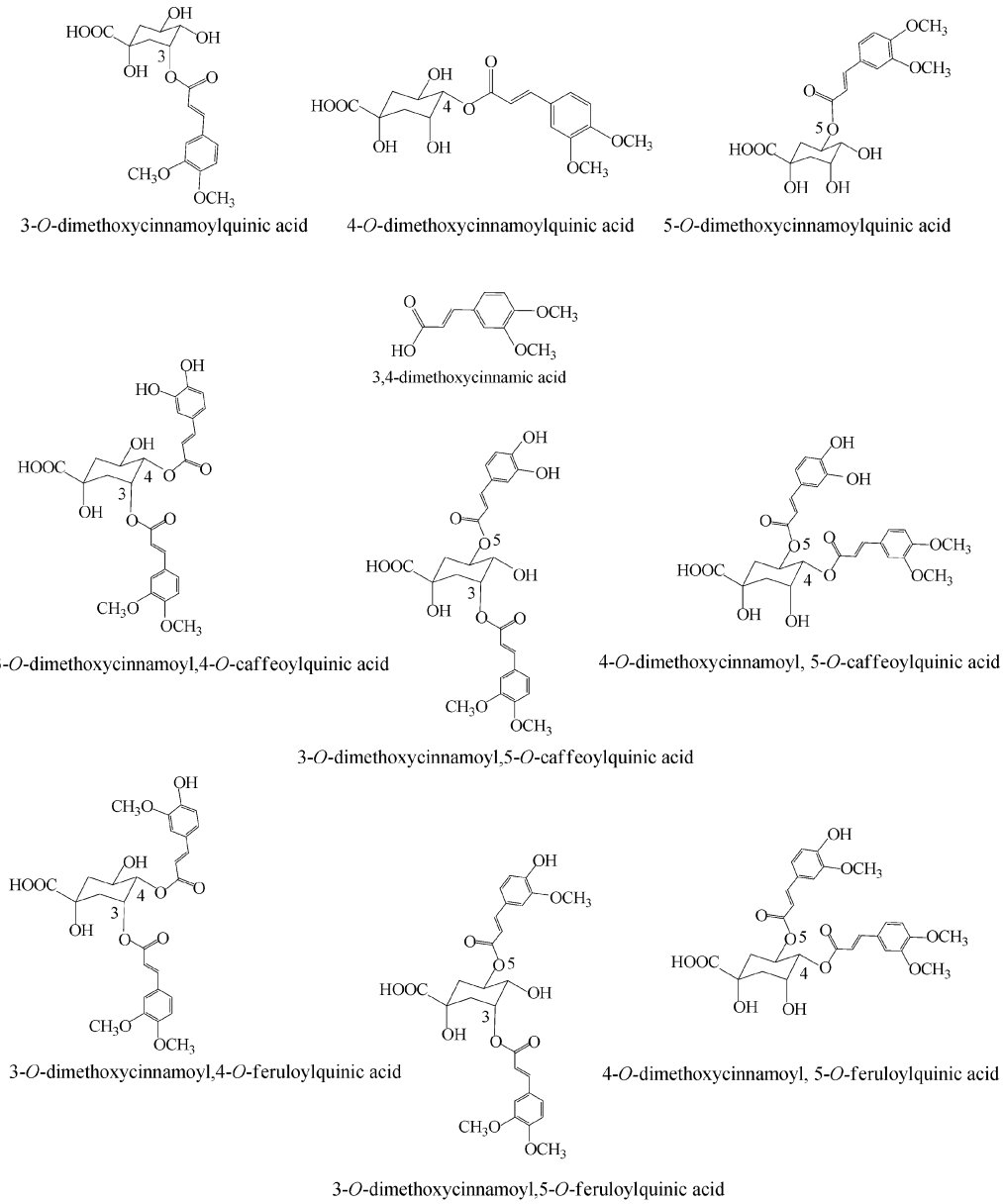

Figure 1. Structures of known quinic acid esters of dimethoxycinnamic acid in green coffee beans.

(20 μL) was added to 380 μL of blank plasma and submitted to the plasma extraction procedure described in Section 2.5, with a final concentration of 150, 300, or 600 nM. The plasma extraction was performed in triplicate on three separate days and the amount of dimethoxycinnamic acid in the spiked sample was determined by HPLC-MS^2. The mean recovery from plasma, expressed as a percentage of the initial spike amount, were 105 ± 6, 99 ± 4, and 94 ± 7% for reference standards 150, 300, or 600 nM, respectively.

2.9 Quantification

Reference samples for the quantification of analytes in coffee solution and cell culture samples were prepared by dissolving a high concentration of dimethoxycinnamic acid in DMSO and diluting in 5% ACN: water (5:95, v/v) containing sinapic acid as internal standard. Known amounts were analyzed by HPLC-MS^2 in MRM mode as described above. The final concentration of internal standard was 6 μM and DMSO was maintained at 0.2% in all reference samples. The peak area for the base peak (*m/z* 103) of each reference sample was plotted against dimethoxycinnamic acid concentration (nM) and used to quantify the concentration of dimethoxycinnamic acid and DQAs in the instant coffee samples and culture solutions.

For the quantification of analyte in human plasma, a calibration curve was constructed by adding 20 μL of reference sample containing 3-(4-hydroxyphenyl)-propionic acid as the internal standard to 380 μL of blank plasma, which was submitted to the plasma extraction procedure described in Section 2.5 in the absence of chlorogenate esterase. Reference samples were reconstituted in 100 μL of 5% ACN: water (5:95, v/v) and the filtrates were analyzed by HPLC-MS^2 in MRM mode. The calibration curve had a final concentration of 150, 300, 600, 1200, 2400, and 4800 nM dimethoxycinnamic acid and the internal standard was maintained at 500 nM. All solutions were prepared fresh on the day of analysis.

2.10 Statistics

Analysis of variance or a Dunnett's pairwise multiple comparison *t*-test, when comparing a set of treatments to a control, was used to confirm statistical difference in samples under different experimental conditions and is a test of whether the means of two or more groups are equal. Shilpro–Wilk and Levene's test were performed to confirm the normality of the data and the equality of variances, respectively. The mean difference was statistically significant at the 5% level. Linear dependence was investigated by analysis of the coefficient of determination (R^2) and confirmed by Pearson's correlation coefficient, which was significance at the 1% level (PASW statistics (v 17)).

3 Results

3.1 Detection of analytes in coffee solution

The composition of the decaffeinated instant coffee used in the cell culture works was in line with that consumed in the human study (Table 1.) The profile of DQA derivatives was investigated by HPLC-MS^2, and Fig. 1 shows those which have been identified in green coffee beans. A typical MS trace of the coffee solution and the major corresponding product ion in MRM mode can be seen in Fig. 2, showing that the coffee solution contained three DQA isomers with negatively charged molecular ions $[M\text{-}H]^-$ at *m/z* 381, eluting between 30 and 45 min. Peak 1 (R_t 33.5 min) yielded a major MS^2 ion (base peak) at *m/z* 207 and a smaller ion at *m/z* 173 (3% of base peak). The *m/z* 207 ion corresponds to the dimethoxycin-

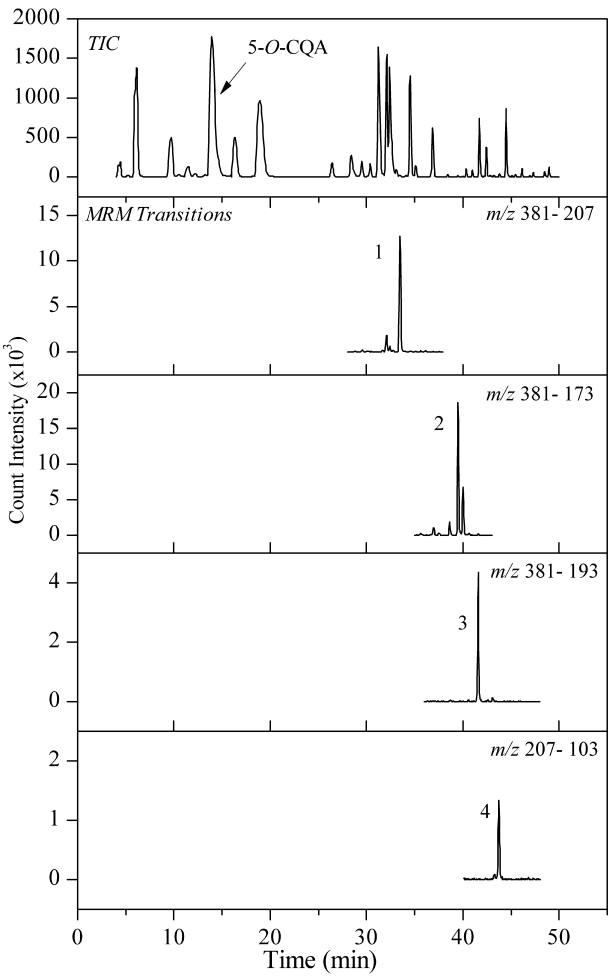

Figure 2. HPLC-MS^2 traces of dimethoxycinnamoylquinic acid esters and free dimethoxycinnamic acid detected in instant coffee. TIC = total ion count trace of coffee solution; MRM transitions: (1) 3-*O*-dimethoxycinnamoylquinic acid; (2) 4-*O*-dimethoxycinnamoylquinic acid; (3) 5-*O*-dimethoxycinnamoylquinic acid; (4) 3,4-dimethoxycinnamic acid.

namic acid ion [DQA + H_2O–quinic acid–H^+]$^-$, with similar behavior to 3-*O*-feruloylquinic acid as previously reported [2] and was assigned as 3-*O*-DQA. Peak 2 (R_t 39.5 min) had ion products at *m/z* 173 and 207 corresponding to the quinic acid fragment [DQA–dimethoxycinnamic acid–H^+]$^-$and the dimethoxycinnamic acid ion, respectively, and so the isomer was assigned as 4-*O*-DQA because of the analogous fragmentation to 4-*O*-feruloylquinic acid and reported data for 4-*O*-DQA [17]. Peak 3 (R_t 41.6 min) occurred in lower amounts and formed MS^2 fragment ions at *m/z* 193 and 173, which are in agreement with the reported fragmentation mechanism for 5-*O*-DQA leading to a ketene quinide fragment [DQA + H_2O–quinic acid–methyl cation]$^-$ and a dehydrated quinic acid fragment, respectively [17]. Peak 4 (R_t 43.7 min) had an [M-H]$^-$ at *m/z* 207, which yielded MS^2 ions at *m/z* 103 and 131 characteristic of dimethoxycinnamic acid, and so the fragments are postulated as corresponding to [dimethoxycinnamic acid–COOH–2(OCH_3) + 2(H^+)]$^-$ and an alkyne fragment [14], respectively.

3.2 Caco-2 cell permeation

The permeability of dimethoxycinnamic acid and DQAs across differentiated Caco-2 monolayers was determined using HPLC-MS^2 detection of the corresponding *m/z* transitions for 207 and 381, respectively, without enzymatic deesterification. The decaffeinated coffee solution (18 mg/mL) used to investigate membrane permeation containing 1.8 μM of free dimethoxycinnamic acid and 2.2 μM total DQAs, which were low in comparison to 5-*O*-CQA (1 mM), Table 2. No DQA isomers were detected in the basal chamber following apical incubation with the coffee solution. In contrast, a substantial amount of dimethoxycinnamic acid was transported across the membrane in the 40-min incubation period, which was ten times more than 5-*O*-CQA. In addition, Caco-2 monolayers demonstrated esterase activity, leading to a significant ($p < 0.05$) increase in the total amount of free dimethoxycinnamic acid detected in apical and basal chambers following incubation of coffee solution (Table 2). Calculation of the permeability coefficient (P_{app}) revealed that membrane permeation is rapid, possibly attributed to a combination of small molecular size (208 Da) and moderate lipophilicity. Permeation of dimethoxycinnamic acid showed time-dependent uptake, and quickly formed a plateau when 50% of the apical dose had been transported to the basal compartment (Fig. 3A). Furthermore, the transport was linear with respect to concentration over the concentrations achievable using coffee solution indicating passive absorption (Fig. 3B).

A synthetic standard of 3,5-diDQA (500 μM) was used as a model compound to explore the permeability of DCiQAs (Table 3). During this investigation, a higher permeability was observed for analytes transported in six-well than in 12-well plates, which is associated with lower membrane resistance of large area inserts (user guide, Millipore Ltd.). Thus, the permeation of 3,5-diDQA is presented alongside 5-*O*-CQA at an equivalent concentration for ease of comparison. Interestingly, despite the favorable lipophilicity (Log *D* –0.6), permeation of 3,5-diDQA was shown to be similar to that of 5-*O*-CQA, 0.18 and 0.38 cm/s, respectively.

3.3 Plasma pharmacokinetic profile of dimethoxycinnamic acid derivatives

Measurements of metabolites in plasma were taken between 0 and 24 h following ingestion of instant coffee containing 900 μmol total CGAs. Pharmacokinetic profiles for the mean plasma concentration of dimethoxycinnamic acid and dimethoxy-dihydrocinnamic acid after treatment with an enzyme cocktail containing β-glucuronidase, sulfatase, and

Table 2. Permeation of dimethoxycinnamic acid derivatives in decaffeinated instant coffee solution (18 mg/mL) across differentiated Caco-2 monolayers

Compound	Apical dose nmol	Amount		Total nmol	P_{app} cm/s	Log$D_{pH\ 7.4}$
		Apical nmol	Basal nmol			
3,4-dimethoxycinnamic acid	0.9 ± 0.2	0.8 ± 0.04	0.9 ± 0.1	1.7 ± 0.14[c)]	181 ± 16	−1.01[a)]
3-*O*-dimethoxycinnamoylquinic acid	0.53 ± 0.04	0.5 ± 0.05	n.d		-	−3.28[b)]
4-*O*-dimethoxycinnamoylquinic acid	0.42 ± 0.07	0.4 ± 0.03	n.d		-	−3.27[b)]
5-*O*-dimethoxycinnamoylquinic acid	0.13 ± 0.05	0.12 ± 0.01	n.d		-	−3.28[b)]
5-*O*-caffeoylquinic acid	523 ± 9.4	488 ± 47.9	0.1 ± 0.01	488.1 ± 48	0.04 ± 0.0	−3.05[a)]

Values are expressed as amount (nanomole) ± SD ($n = 3$) in apical and basal volumes of 0.5 and 1.5 mL, respectively. P_{app} = permeability coefficient, values expressed in cm/s × 10^{-6}. n.d = not detected. Log *D* = distribution coefficient at pH 7.4.
a) Values from [21].
b) Values from [22].
c) A statistical difference was observed following incubation (40 min) of coffee solution in the presence of Caco-2 cells ($p < 0.05$).

esterase are presented in Fig. 4C. The bioavailability was estimated to be 143.6 ± 23.5 and 46.0 ± 6.5 μmol/h, respectively, based on AUC calculation of the amount absorbed.

Dimethoxycinnamic acid was present in the plasma of all volunteers and reached the C_{max} within 1 h after coffee intake. The pharmacokinetic profile indicated an early appearance (0–1 h) in plasma followed by a gradual decline over the next 2–24 h, Fig. 4A. In three volunteers, a biphasic curve in plasma was observed (Fig. 4B) with a second smaller peak between 2 and 4 h postingestion. In contrast, dimethoxydihydrocinnamic acid appeared in plasma above baseline predominantly 8–12 h following coffee consumption with a mean C_{max} value of 97 ± 27 nM, Fig. 4A. Interestingly, some volunteers displayed an early appearance in plasma within 1 h postingestion, accompanied by an increasing concentration at 12 h as shown in Fig. 3B. In all cases, the plasma concentration of metabolites returned to baseline at 24 h.

3.4 Quantification of dimethoxycinnamic acid in human plasma

The amounts of dimethoxycinnamic acid and DQAs in human plasma were investigated by HPLC-MS^2 in the absence of chlorogenate esterase to explore the native structures in circulation. Due to the complexity of structures and the absence of standards for DCiQAs, these compounds were not investigated by HPLC-MS^2. Instead, enzymatic de-esterification was performed to allow quantification of the total amount of dimethoxycinnamic acid from all potential dimethoxycinnamic acid derivatives (Fig. 1). No dimethoxycinnamic acid or DQAs were detected in baseline plasma after submission to the plasma extraction procedure. Furthermore, no DQAs could be detected in plasma following processing in the absence and presence of enzymatic de-esterification. These results suggest that DQAs and DCiQAs are not present as a major component in plasma. Free dimethoxycinnamic acid was the only form detected in plasma samples and the mean C_{max} was quantified as 496 ± 110 nM ($n = 8$) (Table 4). Furthermore, no statistical difference in the mean plasma concentration ($n = 8$) of dimethoxycinnamic acid for before and after enzymatic de-esterification was observed. The interindividual variability in bioavailability appears to be relatively small (20%) as concluded by the low SD of the mean plasma concentration, ranging from 405 to 677 nM ($n = 8$). Similarly, the modal T_{max} value for dimethoxycinnamic acid was 30 min, with just two instances at 60 and 90 min, suggesting absorption in the upper gastrointestinal tract.

4 Discussion

Intestinal absorption of dimethoxycinnamic acid derivatives has never been investigated before. In the current study, an optimized methodology was applied to the detection and quantification of dimethoxycinnamic acid derivatives after cellular transport, without enzymatic de-esterification. Small amounts of DQA esters and free dimethoxycinnamic acid were detected in the instant coffee at low concentration (<2 μM). The cell membrane permeability of dimethoxycinnamic acid was shown to be ten times greater than 5-*O*-CQA, and no DQAs could be detected on the serosal (basal) side. Furthermore, significant amounts of DQAs and DCiQAs were hydrolyzed resulting in a 90% increase of the free dimethoxycinnamic acid content following incubation of instant coffee with differentiated Caco-2 monolayers. These results suggest that following ingestion of coffee, the small intestine may be an important site for hydrolysis of dimethoxycinnamic acid derivatives by intestinal esterases

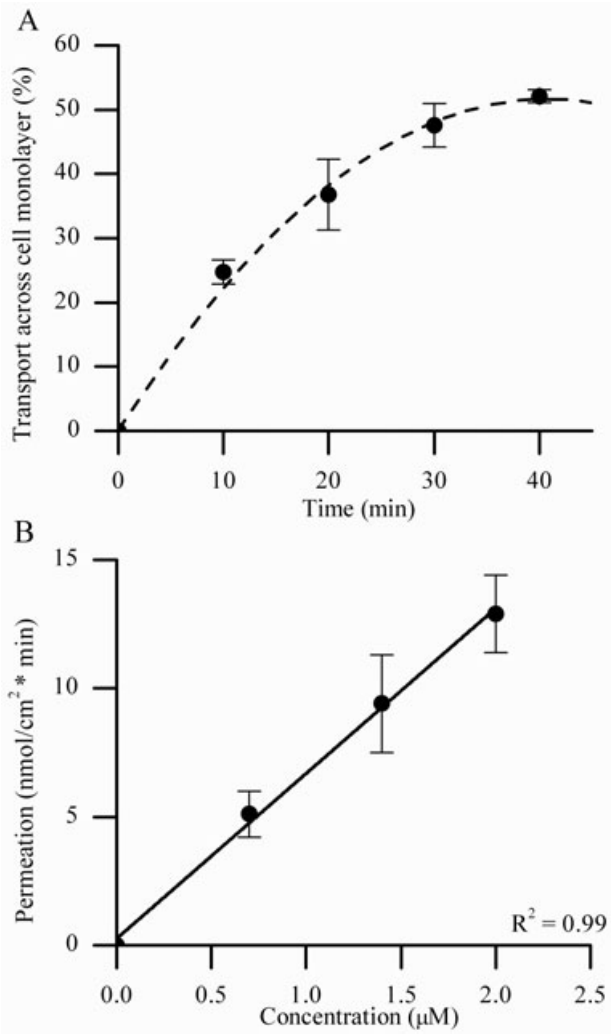

Figure 3. (A) Time-dependent permeability of dimethoxycinnamic acid expressed as the percentage of the apical dose detected in the basal compartment over a 40-min period. Dashed line indicates nonlinear relationship. (B) Concentration-dependent permeability. Permeation of dimethoxycinnamic acid across differentiated Caco-2 monolayers was measured by detection in the basal compartment after 30-min incubation with three concentrations of coffee solution (9, 18, and 27 mg/mL). Values are the mean of three cultures ± SD.

Table 3. Permeation of 5-*O*-caffeoylquinic acid and 3,5-di-*O*-dimethoxycinnamoylquinic acid reference standards across differentiated Caco-2 monolayers

Compound	Apical dose nmol	Amount		P_{app} cm/s	Log*D* $_{pH\ 7.4}$
		Apical nmol	Basal		
5-*O*-caffeoylquinic acid	1054 ± 0.4	1000 ± 60	3.4 ± 1.0	0.38 ± 0.1	−3.05[a)]
3,5-di-*O*-dimethoxy-cinnamoyl quinic acid	1000 ± 1	616 ± 17	1.5 ± 0.6	0.18 ± 0.1	−0.57[b)]

Values are expressed as amount (nanomole) ± SD detected in apical and basal compartments (2 mL) after separate incubations with 500 μM of compound. Experiments were performed using triplicate cultures between passages 35–45.
a) Values from [21].
b) Values from [22].

[23], which have been characterized previously in Caco-2 cell monolayers and human intestinal tissue [24].

Dimethoxycinnamic acid, as present in coffee solution, is preferentially absorbed in the free form by passive absorption as determined by the linearity of transfer over time, unsaturated uptake at the highest concentration we could achieve and we have previously shown the bidirectional transport to be equivalent [25]. An explanation for the differential absorption of dimethoxycinnamic acid aglycone compared with the quinic acid esters may be its small molecular weight and relative lipophilicity, which favor transcellular transport [26]. The relationship between these physicochemical attributes and absorption has been described previously [25] and indicate that poor lipophilicity and increased molecular size have particular importance to diffusion across biological membranes [26] by restriction of membrane partitioning and diffusivity, respectively. These results suggest that transport across the intestinal barrier is a rate-limiting step for the DQA derivatives.

The identification of dimethoxycinnamic acid in human plasma of coffee drinkers, following treatment with an enzyme cocktail, was recently reported [14]. Here, we have shown that dimethoxycinnamic acid is present in human plasma within 30 min following ingestion of instant coffee in the free form and with low interindividual variability. Enzymatic hydrolysis was used to investigate the presence of DCiQAs by proxy through the identification of DQAs after enzymatic de-esterification of the plasma samples. Interestingly, no DQAs were detected before or after enzyme treatment. Furthermore, the absence of DQAs in plasma is supported by data from a preliminary study on one volunteer after ingestion of a 400-mL cup of coffee [27]. Thus, we concluded that either dimethoxycinnamic acid was the only form in plasma or that 3,4-*O*- DCiQAs were present, but because our enzyme is specific for hydrolyzing the 5-*O*-acyl position, we were unable to detect them. Our plasma profiles of the dimethoxycinnamic acid derivatives reflect their absorption characteristics in the intestinal model as discussed above.

Determination of the C_{max} value of dimethoxycinnamic acid in plasma, in the absence of enzyme hydrolysis, has never been performed before for a cohort human study. Only

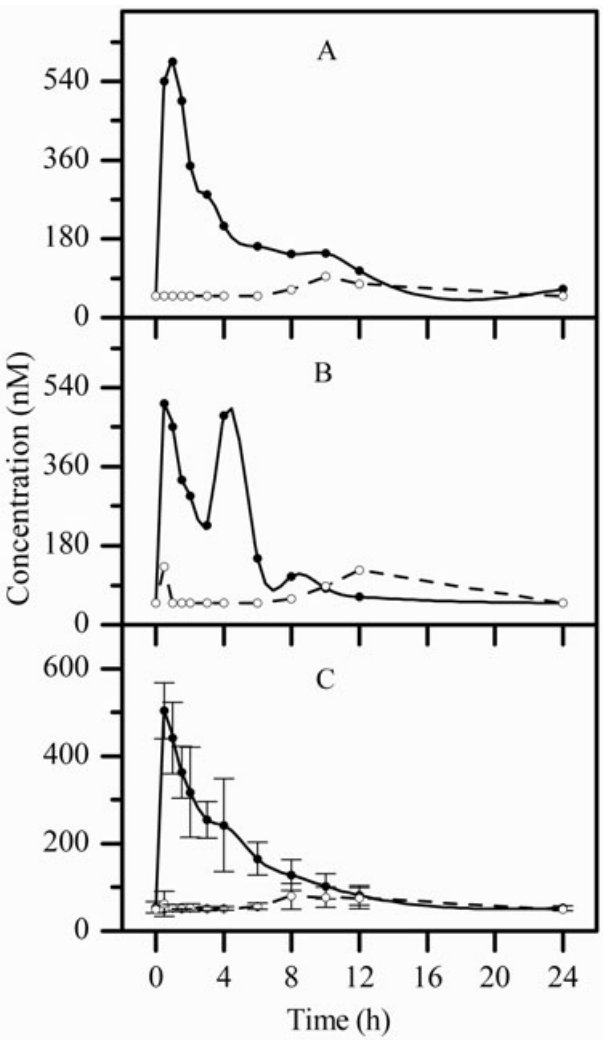

Figure 4. Plasma pharmacokinetic profile of dimethoxycinnamic acid (bold line) and dimethoxy-dihydrocinnamic acid (dashed line), following ingestion of 400 mL of instant coffee. (A) Typical absorption profile. (B) Biphasic absorption of dimethoxycinnamic acid and early (1 h) appearance of dimethoxy-dihydrocinnamic acid was observed in some volunteers. (C) Mean pharmacokinetic profile of eight volunteers.

Table 4. Quantification of 3,4-dimethoxycinnamic acid at the C_{max} time point in human plasma with and without enzyme hydrolysis treatment

Extraction procedure	Volunteer								Mean ± SD
	1	2	3	4	5	6	7	8	
	nM								
No enzyme treatment	451 ± 2	485 ± 32	405 ± 1	553 ± 20	677 ± 14	614 ± 29	433 ± 35	349 ± 9	496 ± 110
0.1 U chlorogenate esterase	475 ± 17	526 ± 31	364 ± 30	563 ± 7	702 ± 36	602 ± 29	433 ± 29	393 ± 16	507 ± 114

Plasma C_{max} values for individual volunteers, expressed as mean ± SD ($n = 2$) in nM. No significant difference in plasma concentration of dimethoxycinnamic acid was observed between treatment conditions ($p = 0.448$).

one other bioavailability study has reported early appearance of dimethoxycinnamic acid in human plasma [14] and their detection of dimethoxycinnamic acid at 1 h after consumption of a similar coffee beverage was highly comparable to the amounts quantified in the current study (around 400 nM). A few human studies have determined the amounts of individual early appearing HCAs in plasma of coffee drinkers [28,29]. The reported data suggested that the typical C_{max} value was less than 100 nM occurring mainly as sulfated conjugates. The substantial amount of unconjugated dimethoxycinnamic acid in human plasma determined in this study was more closely comparable to the amount of sulfated conjugates of dihydrocaffeic acid (325 ± 99 nM) and dihydroferulic acid (385 ± 86 nM) which, until now, were the major metabolites identified human plasma after drinking a typical coffee beverage [28,29].

Analysis of the pharmacokinetic data for dimethoxycinnamic acid revealed a short T_{max} value for dimethoxycinnamic acid, which infers absorption may also occur in the stomach. This result supports earlier studies by our group using in vitro gastric mucosal monolayers [21]. The authors demonstrated that dimethoxycinnamic acid derivatives, as present in coffee, are efficiently de-esterified and a significant amount of dimethoxycinnamic acid was transported to the serosal side. In this current study, smaller amounts of dimethoxy-dihydrocinnamic acid were quantified in plasma, approximately 8–12 h after coffee intake. The late appearance was presumably attributed to the conversion of dimethoxycinnamic acid to its reduced form by colonic microfloral reductase. In some cases, dimethoxy-dihydrocinnamic acid was present within 1 h after coffee consumption, possibly attributed to intestinal epithelial reductase activity. Intestinal release of the reduced phenolic acids has been previously reported following incubation of ferulic acid with confluent Caco-2 cells [21,30]. These data suggest that both dimethoxycinnamic acid and its reduced form are highly bioavailable.

In conclusion, the Caco-2 mechanistic data suggest that dimethoxycinnamic acid is transported across the intestinal membrane by passive diffusion, while DQAs could not be detected in the basal (serosal) side. After consumption of coffee, DQAs are probably subject to the action of intestinal esterases, leading to liberation of dimethoxycinnamic acid, which is absorbed and appears at a high abundance in human plasma. Importantly, the pharmacokinetic data show, for the first time, that the dimethoxycinnamic acid is present in circulation within 30 min and there is low variability in the maximum plasma concentration. This study shows that dimethoxycinnamic acid, previously detected in human plasma after coffee intake, is present in the free form.

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