Journal of Pharmaceutical and Biomedical Analysis 55 (2011) 1245–1254

Contents lists available at ScienceDirect

Journal of Pharmaceutical and Biomedical Analysis

journal homepage: www.elsevier.com/locate/jpba

Characterization of hydroxycinnamic acid glucuronide and sulfate conjugates by HPLC–DAD–MS^2: Enhancing chromatographic quantification and application in Caco-2 cell metabolism

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ARTICLE INFO

Article history:
Received 7 February 2011
Received in revised form 11 March 2011
Accepted 14 March 2011
Available online 21 March 2011

Keywords:
LC–MS/MS
Phenolic acid
Sulfation
Bioavailability
Caco-2
Phase II metabolism

ABSTRACT

Hydroxycinnamic acids (HCAs) are emerging naturally occurring anti-inflammatory bioactive compounds. Determination of HCA metabolism has been restricted by the lack of authentic standards for the *in vivo* metabolites, and so the bioavailability of metabolites is often estimated post-hydrolysis. Recently a set of HCA conjugates were chemically synthesized allowing their detection in biological fluids in a very limited number of studies. However, authentic standards are not widely available and for many investigators accurate quantification of HCA conjugates remains a major analytical challenge. Consequently, we have characterized novel physicochemical properties of 14 authentic standards of HCA conjugates; the resulting data will permit for the first time the accurate quantification of HCA conjugates relative to the parent aglycone without the need for standards. MS operating conditions were optimized to achieve excellent sensitivity, and limits of detection by MS ranged from 3 to 15 nM for 12 out of 14 conjugates and 30 to 50 nM for the remaining. Intra-day and inter-day precision and accuracy was calculated at $<\pm 6\%$ and $<\pm 10\%$ respectively. For the first time response factors were determined by triple-quadrupole MS and spectroscopic detection methods, providing essential correction factors. Moreover, we present original analysis of UV-absorbance spectral shifts for HCA conjugates with regio-isomerization, which is advantageous for their differentiation. We demonstrate the usefulness of this method to assess the fate of hydroxycinnamic acids in the Caco-2 cell intestinal model and the impact of metabolism on HCA physicochemistry. For the first time, four HCA conjugates have been unequivocally identified as novel Caco-2 monoculture intestinal metabolites: ferulic acid-4-*O*-glucuronide, dihydroferulic acid-4-*O*-sulfate, caffeic acid-4-*O*-sulfate, and caffeic acid-3-*O*-sulfate. The characterization data presented here will significantly improve quantification and understanding of the bioavailability *in vivo*.

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1. Introduction

Hydroxycinnamic acids (HCAs) are a subgroup of phytochemicals occurring widely in the plant kingdom [1] which have been explored as anti-carcinogenic agents [2], and are active components of herbal medicines, especially *Cynara cardunculus* [3] and *Ilex latifolia* [4]. The major dietary source of HCA is coffee, where most commonly caffeic acid, ferulic acid, or *p*-coumaric acids are linked to a quinic acid moiety to form a family of esters known as chlorogenic acids (CGAs) [1]. This family of compounds has been associated with reduced risk of type 2 diabetes [5], and several potential mechanisms of efficacy [4,6] have been proposed. Recent identification of hydroxycinnamic acids and their conjugates as the major circulating forms following coffee consumption [7] further contributes to the shifting paradigm of polyphenol research towards intestinal, colonic and hepatic metabolites as the principal bioactives [8,9] with distinct biological activities [10].

Analysis of HCA metabolism with several possible positions of conjugation and multiple substitutions has proven a major analytical challenge over many years. Recently a set of 24 potential human metabolites of coffee HCAs have been chemically synthesized [11], allowing a small number of studies to identify an accurate profile of HCA metabolites in human plasma and urine [7]. Further to this, a very few studies have been able to demonstrate the regio-selectivity of Caco-2 cell metabolism of HCAs using authentic standards [12]. Despite the advances in analytical identification of HCA conjugates, the lack of widely available authentic standards is a major limitation for accurate quantification. The majority of studies determine the bioavailability of HCA conjugates

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doi:10.1016/j.jpba.2011.03.023

after β-glucuronidase/sulfatase enzymatic hydrolysis to release the parent hydroxycinnamic acid [13] and consequently important information on the nature of circulating conjugates is lost. Recent assessment of enzymatic efficiency of HCA conjugates hydrolysis in plasma revealed recoveries of <93% and 24% for β-glucuronidase and sulfatase respectively [14]. The apparent potential for incomplete enzymatic hydrolysis/poor recovery introduces an inherent underestimation of HCA concentration. Alternatively, intact conjugate forms may be confirmed by LC–MS^n and the amount estimated relative to the parent compound [15]. Both methods yield limited information based on indirect quantification using calibration curves of the parental hydroxycinnamic acid. A critical flaw in these techniques is the assumption that the chromatographic response of the conjugate is equivalent to the parent compound. Although valuable, these studies provide an incomplete and inherently inaccurate picture of the bioavailability of the circulating metabolites. Reverse-phase liquid chromatography combined with different detection methods is commonly used to separate and identify hydroxycinnamic acids [16] and most recently their conjugates [7,12,17]. However, the reported analytical methods for quantification of HCA conjugates in the absence of authentic standards are deficient and provide misleading estimates of bioavailability.

Consequently, we have used a common HPLC–DAD–MS^2 methodology to analyze novel physicochemical properties of authentic HCA conjugates, which will permit for the first time the accurate quantification HCA conjugate abundance relative to the parent aglycone. To our knowledge this is the first method to develop MS operating conditions optimized for individual HCA conjugates, achieving excellent limits of detection that are up to 15 times more sensitive than previously reported [7]. Moreover, we present original analysis of UV-absorbance spectral shifts for HCA conjugates with regio-isomerization. In previous analytical methods, regio-isomers of HCA conjugates produce identical fragmentation fingerprints under MS analysis [7], and therefore detection is distinguished based on retention time only. Our characterization of the novel UV absorbance properties will facilitate the differentiation between *meta* and *para* positions of conjugation which overcomes the potential ambiguity and variability of retention times between analytical systems.

The validity of the method for use in pharmacological analysis of biological fluids was demonstrated through a pharmacokinetic investigation of Caco-2 cell metabolism of HCAs, with the aim of unequivocal identification and quantification of the main metabolites released by the Caco-2 model of the intestinal epithelium. The impact of intestinal metabolism on HCA physicochemistry was also investigated using modified shake flask and *in silico* techniques providing a unique insight in to the membrane interactions of these potentially bioactive agents. The characterization data presented here will significantly improve the accuracy of HCA conjugate measurement in pharmacological investigations into the bioavailability of these potentially bioactive conjugates, a principal component responsible for *in vivo* efficacy.

2. Materials and methods

2.1. Chemicals

All chemicals reported in this study were purchased from Sigma–Aldrich (Dorset, UK) unless stated otherwise. All water refers to deionized Millipore water, Millipore UK Ltd (Hertfordshire, UK). Culture flasks, Transwell® polycarbonate semipermeable membranes of pore size 0.4 μm and area 4.67 cm^2 were obtained from Corning Life Sciences. Isoferulic acid was purchased from Extrasynthese (Lyon, France). Authentic standards of 3- and 4-*O*-glucuronides and 3- and 4-*O*-sulfates of caffeic, dihydrocaffeic, dihydroferulic, ferulic and isoferulic acids were synthesized as described previously, all conjugates were at least 94% pure [11] and presented data have been corrected for purity.

2.2. Stability of hydroxycinnamic acid conjugates at 4 °C storage

To assess stability at 4 °C, HCA aglycones, glucuronides and sulfates were reconstituted with 5% acetonitrile in water (5:95, v/v) and ascorbic acid (final concentration 1 mM). Standards were combined in distinct groups; sulfates (75 μM), glucuronides (40 μM), and HCA (85 μM) and refrigerated (4 °C) for 7 d ($n = 2$). The initial concentration was determined by HPLC–DAD to be >95% of the expected value and the percentage of initial concentration remaining was determined at 7 d storage (4 °C).

2.3. Recovery efficiency for transport studies

Extraction efficiency experiments were performed at pH 6 and 7.4 for caffeic, ferulic, isoferulic, dihydrocaffeic and dihydroferulic acids and the recoveries were used as a correction factor in quantifying their glucuronide and sulfate forms. In brief, HCA aglycones were prepared from a concentrated stock in DMSO and diluted to 10 μM in HBSS at pH 6 or pH 7.4, where final percentage of DMSO was 0.2%. Samples were incubated for 2 h, in the absence of cells, in 12 well Transwell plates. After incubation, a portion (50 μl) was removed, deproteinated as described below and recovery analyzed by HPLC–DAD ($n = 5$).

2.4. HPLC–DAD–MS optimization and compound characterization

HCA glucuronide and sulfate standards were dissolved in 5% acetonitrile in water (5:95, v/v) and injected on to a Rapid Resolution HPLC with diode array (DAD) (1200 series Agilent Technologies, Dorset, 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 60 min gradient of (A) premixed 5% acetonitrile in water (5:95, v/v) and (B) premixed 5% water in acetonitrile (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 16% over the next 21 min and immediately increased to 100% for 5 min before initial starting conditions were resumed for a 15 min column re-equilibration.

The HPLC–DAD system was connected to an Agilent 6410 Triple Quadrupole MS with an electrospray ionization (ESI) interface (Agilent Technologies, Dorsert, UK). MS optimization was performed by post-column infusion of individual pure authentic standards in mobile phase directly from the LC to the ESI probe without splitting at a 0.26 ml/min flow rate. Fragmentor voltage was adjusted for each standard to ensure optimal transmittance into the mass analyzer and response of the analyte was monitored in selected ion monitoring (SIM) mode. Similarly a range of collision energies were assessed in multiple reaction monitoring (MRM) mode and we selected the condition which produced the highest abundance of distinct product ions for each compound. MS characterization of the sulfate and glucuronide conjugates was achieved by selection of the $[M-H]^-$ molecular ion in negative ionization mode and identifying the corresponding MS^2 product ions. In the final methodology, HCAs, glucuronides and sulfates were detected in MRM mode based on retention time (R_t) and distinct MS^2 fragment pattern and quantified based on the major MS^2 product ion (base peak) using calibration curves for the corresponding HCA, glucuronide and sulfate standards. Samples were analyzed in negative ionization mode and operation parameters were: nebuliser 60 psi,

drying gas flow 10 l/h, capillary temperature 350 °C, source voltage 4 kV.

To investigate UV absorbance spectra, each conjugate (2.5 μM) was analyzed as described and detected using HPLC–DAD and a corresponding absorbance spectra was extracted from the HPLC–DAD trace. For practical reasons the presented ultraviolet (UV) spectra were determined on column as eluted by up to 10% acetonitrile in water (10:90, v/v). However, polar solvents are known to solvate lone pair electrons and cause spectra shifts in absorbance due to the chromophore moieties within the molecule [18]. Therefore, due to the variation in solvent composition during elution, comparable solutions of conjugates were prepared in 5% acetonitrile in water (5:95, v/v) and the maximal wavelength recorded by absorbance spectroscopy (Cecil Instruments Ltd, UK) to confirm the stated maximum wavelength (Table 3).

2.5. Linearity, precision and accuracy

Method validation was performed according to ICH recommendations and the European Commission Directive for the performance of analytical methods [19,20] including linearity, precision and accuracy, which are principal components of quantification. Linearity was investigated by analysis of peak area response verses concentration over a range of 15 nM to 600 μM for caffeic acid and ferulic acid and from a minimum concentration of 3 nM to 5 μM for conjugate standards. Linear dependence was confirmed by Pearson's correlation coefficient, which was significant at the 0.05 level (PASW statistics. 17). For calibration curves, peak area of the MS^2 base peak ion was plotted against on-column amount and analysis was performed on 2 separate occasions with triplicate injections of each concentration. Precision and accuracy were evaluated for caffeic acid-4-*O*-glucuronide prepared in 5% acetonitrile in water (5: 95, v/v) at 3 quality control (QC) concentrations (15 nM, 200 nM and 2.5 μM); the lower QC level is approximately 2× LLOQ of caffeic acid-4-*O*-glucuronide and is representative of the lower concentration of metabolites detected in our biological samples, and the high QC level is near the upper boundary of the standard curve. Intra-day precision and accuracy was calculated from triplicate injections of the 3 concentrations on the same day. Inter-day precision and accuracy was determined by analysis of triplicate injections of the 3 concentrations on the 3 separate days. Values for precision are expressed as relative standard deviation (R.S.D.) and relative error (R.E.) for accuracy.

2.6. Conjugate response factor on HPLC–DAD and HPLC–DAD–MS^2

The chromatographic response of an analyte is indicative of the amount detected on the column. The response factor, the measure of the ratio of the signal response to the actual amount, was investigated by HPLC–DAD and HPLC–DAD–MS^2 in MRM mode. A small amount of each authentic standard (2.5 μM) was injected onto HPLC–DAD–MS^2 as described above. The peak height at maximum wavelength was determined from the HPLC–DAD chromatogram and compared to the peak height of the corresponding parent aglycone, and data are presented as relative peak height ($n = 2$). The same sample was subsequently analyzed in HPLC–DAD–MS^2 in MRM mode and the MS^2 base peak ion for conjugate and corresponding parent aglycone at an equal concentration were extracted from the MRM trace. The peak height for conjugate MS^2 base peak ion is expressed relative to the peak height for the corresponding parent aglycone MS^2 base peak ion. Response by DAD and MRM mode was corrected for purity by comparing spectroscopic absorbance to aglycone at equal concentration. Corrected values were then normalized to sinapic acid as the internal standard.

2.7. Determination of distribution coefficient (Log *D*) and dissociation constant

Log *D* values of caffeic acid, 5-*O*-caffeoylquinic acid, 3,5-*O*-dicaffeoylquinic acid, dihydrocaffeic acid, dihydroferulic acid, dimethoxycinnamic acid, ferulic acid, 5-*O*-feruloylquinic acid, isoferulic acid, acetylsalicyclic acid, alprenolol, atenolol, hydrocortisone, and metaprolol were determined based on an official validated shake flask method [21]. In order to demonstrate the reliability of *in silico* measurements at more lipophilic values, Log *D* determinations were performed for aromatic compounds to enable comparison of experimental and *in silico* measurements over a broader and more complete range of Log *D* values (−4 to 2). In brief, Bis–Tris buffer was prepared at pH 7.4 (50 mM) and modified with KCl (0.15 M) to create the aqueous solvent. Phases of the solvent system were mutually saturated by shaking equal volumes of *n*-octanol and aqueous solvent at 37 °C on a mechanical shaker for 1 h. Phases were allowed to stand for sufficient time to permit separation and then divided into octanol and aqueous stocks. An aliquot of test compound dissolved in DMSO (0.2% in final volume) was combined with *n*-octanol. An equal volume of aqueous buffer was then added and the partition equilibrium achieved by 100 rotations through 180° in 5 min. Phases were incubated at 37 °C for 1 h; an aliquot (50 μl) was then removed from the *n*-octanol and aqueous phase and the pH of the aqueous phase recorded. Samples were injected on to a Rapid Resolution HPLC–DAD using a Nucleosil C18 column (30 °C, 4.6 mm × 30 mm, 3 μm; Phenomenex). Separation was achieved on an isocratic gradient of (A) premixed 5% acetonitrile in water (5:95, v/v) and 0.1% formic acid for 15 min, then immediately increased to 100% (B) acetonitrile for 5 min before returning to starting conditions for a 5 min column re-equilibration. The test compound was identified by UV spectra and R_t and Log *D* was determined as the mean of 5 replicates. Experimental Log *D* was compared to *in silico* [22]. Due to limited amounts of authentic glucuronide and sulfate standards, Log *D* values and dissociation constant (pKa_1 50% ionization) were calculated [22].

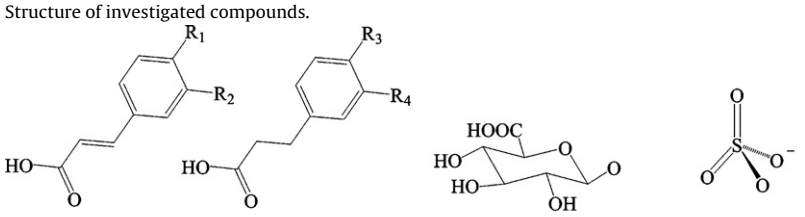
2.8. Cell culture

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 30 and 55. Caco-2 cells were added to transwell inserts at a density 6×10^4 cells/cm^2 and cultured for 21 d at 37 °C under a humidified atmosphere of 95% air: 5% CO_2. DMEM medium 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.

2.9. Transport and metabolism studies

Transport experiments were performed in the presence (pH 6/7.4) and absence (pH 7.4/7.4) of a pH gradient and pH measurements were recorded on the initial transport solution and repeated following the required incubation period. The transport solution was HBSS containing 1.8 mM calcium chloride and adjusted to pH 6 or pH 7.4. A concentrated stock of HCA in DMSO was diluted in transport solution by 500-fold and DMSO was maintained at 0.2% in each transport experiment. On or after 22 d, transport studies were initiated by replacement of growth medium with transport solution; apical pH 6 or pH 7.4 and basal pH 7.4 for 15 min. After equilibration, wells were carefully aspirated to waste and 2 ml HBSS pH 6 or pH 7.4 containing HCA (500 μM) added to the apical donor well and 2 ml HBSS pH 7.4 in the basal receiver well. Transport studies were performed in triplicate at 37 °C under humidified

Table 1
Structure of investigated compounds.



glucuronic acid (glu) sulfate (sul)

Compound	Positional substitution			
	R_1	R_2	R_3	R_4
Aglycones				
Caffeic acid	OH	OH		
Dihydrocaffeic acid			OH	OH
Ferulic acid	OH	OCH_3		
Dihydroferulic acid			OH	OCH_3
Isoferulic acid	OCH_3	OH		
Glucuronides				
Caffeic acid-4-*O*-glucuronide	GlcA	OH		
Caffeic acid-3-*O*-glucuronide	OH	GlcA		
Dihydrocaffeic acid-4-*O*-glucuronide			GlcA	OH
Ferulic acid-4-*O*-glucuronide	GlcA	OCH_3		
Dihydroferulic acid-4-*O*-glucuronide			GlcA	OCH_3
Isoferulic acid-3-*O*-glucuronide	OCH_3	GlcA		
Sulfates				
Caffeic acid-4-*O*-sulfate	sul	OH		
Caffeic acid-3-*O*-sulfate	OH	sul		
Dihydrocaffeic acid-4-*O*-sulfate			sul	OH
Dihydrocaffeic acid-3-*O*-sulfate			OH	sul
Ferulic acid-4-*O*-sulfate	sul	OCH_3		
Dihydroferulic acid-4-*O*-sulfate			sul	OCH_3
Isoferulic acid-3-*O*-sulfate	OCH_3	sul		

atmosphere for 1 h. Thereafter, apical and basal solutions were acidified with acetic acid to a 10 mM final concentration and placed in −80 °C storage.

2.10. Deproteination of culture media

Transport solutions were defrosted and extracted. In brief transport solutions (46 μl) were combined with 6.4 μl of 50% aqueous formic acid, ascorbic acid (final concentration 1 mM) and sinapic acid (final concentration 9 μM) as an internal standard. To the mixture, 113 μl of acetonitrile was added dropwise to precipitate proteins; the samples were vortexed for 1 min and allowed to stand for 1 min, this was repeated 4 times before centrifugation at 17,000 × *g* for 5 min. The supernatant (150 μl) was removed and dried under centrifugal evaporation (Genevac EZ-2 plus, Suffolk, UK). Dried samples were reconstituted (50 μl) in 5% acetonitrile in water (5:95, v/v).

3. Results

3.1. Stability and extraction efficiency

Extraction efficiency of investigated aglycones (Table 1) in HBSS at 37 °C, in the absence of cells, was assessed at pH 6 and pH 7.4. All compounds remained stable in HBSS for 2 h at pH 6 and pH 7.4 (data not shown) and showed no further degradation upon deproteination and centrifugal drying (Table 2). Recoveries were used as correction factors in quantification of the HCA conjugates. The stability of samples prepared for LC–MS analysis was investigated and data indicate minimal degradation following storage under chilled auto-sampler conditions for 7 days (Table 2).

3.2. HPLC–DAD–MS^2 characterization of hydroxycinnamic acid glucuronides and sulfates

The 14 HCA glucuronide and sulfate standards investigated in this study were chemically synthesized and the structures are presented in Table 1. Typical HPLC–DAD–MS^2 chromatograms are presented in Figs. 1 and 2 and showed that all 14 compounds eluted within 30 min using a simple water and acetonitrile gradient. Peaks 1 and 8 are characterized here by HPLC–DAD–MS^2 in MRM mode for the first time; $[M-H]^-$ at *m*/*z* 355 corresponding to caffeic acid-*O*-glucuronide, and confirmed by MS^2 product ion at *m*/*z* 179,

Table 2
Extraction efficiency of hydroxycinnamic acid aglycones from HBSS at 37 °C (2 h) and stability of compounds in acetonitrile: water (v/v 5: 95) after 7 days storage at 4 °C.

Compound	Extraction efficiency % of initial concentration	
	pH 7.4	pH 6
Caffeic acid	96.4 ± 2.3	100.6 ± 5.1
Dihydrocaffeic acid	100.3 ± 3.0	99.6 ± 2.6
Ferulic acid	84.1 ± 1.8	82.4 ± 5.3
Dihydroferulic acid	97.9 ± 4.2	98.3 ± 2.9
Isoferulic acid	95.5 ± 0.7	94.8 ± 6.1

Compound	Stability at 4 °C % of initial concentration
Aglycones	99.4–100.1%
Glucuronides	99.7–100.2%
Sulfates	97.3–102.1%

Values for extraction efficiency are mean ± SD ($n = 4$). Values for stability at 4 °C are the range of recovery for compounds listed in Table 1 ($n = 2$ per standard).

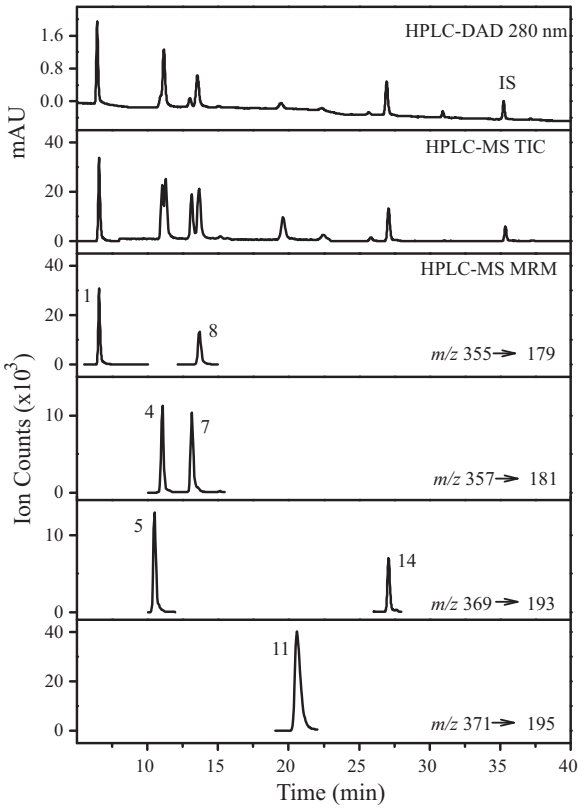


Fig. 1. HPLC–DAD–MS^2 traces of synthetic glucuronide standards. HPLC–DAD–MS^2 traces: (1) caffeic acid-4-*O*-glucuronide, (4) dihydrocaffeic acid-4-*O*-glucuronide, (5) ferulic acid-4-*O*-glucuronide, (7) dihydrocaffeic acid-3-*O*-glucuronide, (8) caffeic acid-3-*O*-glucuronide, (11) dihydroferulic acid-3-*O*-glucuronide, and (14) isoferulic acid-4-*O*-glucuronide at 2 μM.

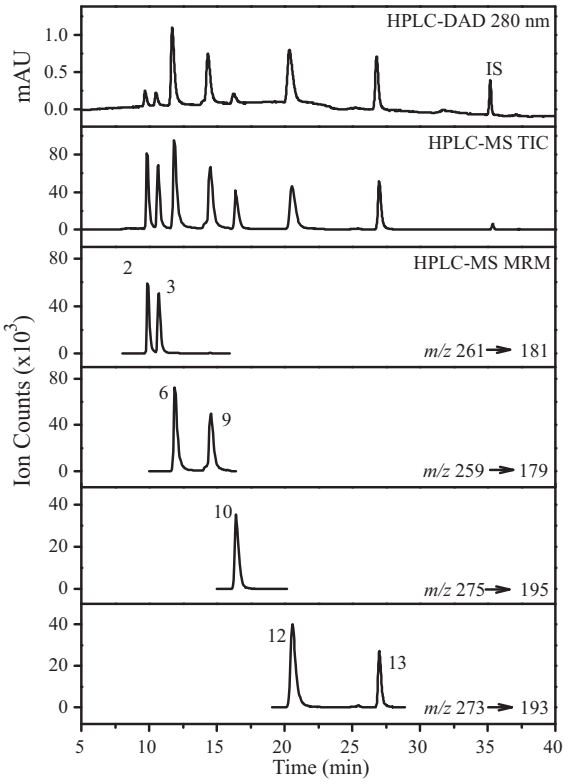


Fig. 2. HPLC–DAD–MS^2 traces of synthetic sulfate standards. HPLC–DAD–MS^2 traces of sulfate standards: (2) dihydrocaffeic acid-4-*O*-sulfate, (3) dihydrocaffeic acid-3-*O*-sulfate, (6) caffeic acid-4-*O*-sulfate, (9) caffeic acid-3-*O*-sulfate, (10) dihydroferulic acid-4-*O*-sulfate, (12) ferulic acid-4-*O*-sulfate, and (13) isoferulic acid-3-*O*-sulfate at a concentration of 2 μM.

135 identified as caffeic acid and [M−COOH]$^-$. Positional substitution was confirmed by the R_t of the authentic standards; caffeic acid-4-*O*-glucuronide (6.6 min) and caffeic acid-3-*O*-glucuronide (13.7 min). Peaks 4 and 7 had [M−H]$^-$ at *m*/*z* 357 corresponding to the isomers of dihydrocaffeic acid-*O*-glucuronide and product ions 181, 175, 113 corresponding to dihydrocaffeic acid, the glucuronide moiety and its product ion [M−H_2O−COOH]$^-$ [7,23]. R_t confirmed the isomers as dihydrocaffeic acid-4-*O*-glucuronide (11.1 min) and dihydrocaffeic acid-3-*O*-glucuronide (13.2 min). Peaks 5 and 14 had [M−H]$^-$ at *m*/*z* 369 characteristic of ferulic acid-4-*O*-glucuronide, on which MS^2 produced *m*/*z* 193 and 113 corresponding to ferulic acid and cleavage ion of the glucuronide [M−H_2O−COOH]$^-$ [7,12]. R_t of authentic standards confirmed peak 14 as isoferulic acid-3-*O*-glucuronide (27.1 min). Peak 11 was identified by the [M−H]$^-$ at *m*/*z* 371 and gave similar product ions as peak 5, *m*/*z* 195 and 113, which indicated the reduced form, dihydroferulic acid-4-*O*-glucuronide [7].

The fragmentation of [M−H]$^-$ in MS^2 was determined for the sulfate conjugates as follows: peaks 2 and 3 had [M−H]$^-$ at *m*/*z* 261 indicative of dihydrocaffeic acid–sulfate and identified by product ions *m*/*z* 181, 137, which correspond to dihydrocaffeic acid and [M−COOH]$^-$ [23]. Peaks 6 and 9 at *m*/*z* 259 yielded MS^2 ions *m*/*z* 179, 135; both ions are indicative of caffeic acid and the net loss of 80 Da is characteristic of a sulfate moiety [7]. Positional isomerisation was confirmed by authentic standards, supported by UV spectral shift to 310 nm for caffeic acid-3-*O*-sulfate (peak 9). Peaks 12 and 13 gave a [M−H]$^-$ at *m*/*z* 273 which produced MS^2 ions at *m*/*z* 193, 178, 134 corresponding to ferulic acid. Authentic standards identified peak 12 as ferulic acid-4-*O*-sulfate (20.6 min) and peak 13 as the isoferulic acid-3-*O*-sulfate (27.0 min). Peak 10 had a [M−H]$^-$ at *m*/*z* 275 corresponding to dihydroferulic acid-4-*O*-sulfate [23] and yielded MS^2 ions at *m*/*z* 195 and 136 corresponding to dihydroferulic acid and [M−COOH]$^-$ and the net loss of 80 Da confirmed the sulfate residue.

3.3. Effect of positional conjugation on absorbance spectra

Absorbance spectra for the authentic standards are presented in Fig. 3. HCA aglycones have similar spectra with the characteristic 320 nm maxima and a 290 nm shoulder. Conjugation in the *meta*-position (3-*O*-) did not alter the absorbance maxima, although a broader apex was observed. Comparatively, conjugation in the *para*-position (4-*O*-) was identified on HPLC–DAD by a distinct shift in absorbance maxima to 280/290 nm.

3.4. Linearity, precision and accuracy

For all compounds detected in Tables 5 and 6, peak area varied linearly with on-column amount over the ranges specified in section 2.5 ($R^2 > 0.99$), and Pearson's correlation coefficient confirmed the linear dependence, which was equal to 1 at a significance level of 0.01. Intra-day and inter-day precision was calculated for caffeic acid-4-*O*-glucuronide as R.S.D. <2.5%, <1.5% and <6% for 15 nM, 200 nM and 2500 nM concentrations respectively. Good intra-day and inter-day accuracy was demonstrated across the concentration range with relative error <±10%. The precision and accuracy meet performance criteria for analytical methods, which indicates precision (R.S.D.) and accuracy (R.E.) must be within ±15%, or for the lower limit of quantification, values within ±20% are acceptable [19].

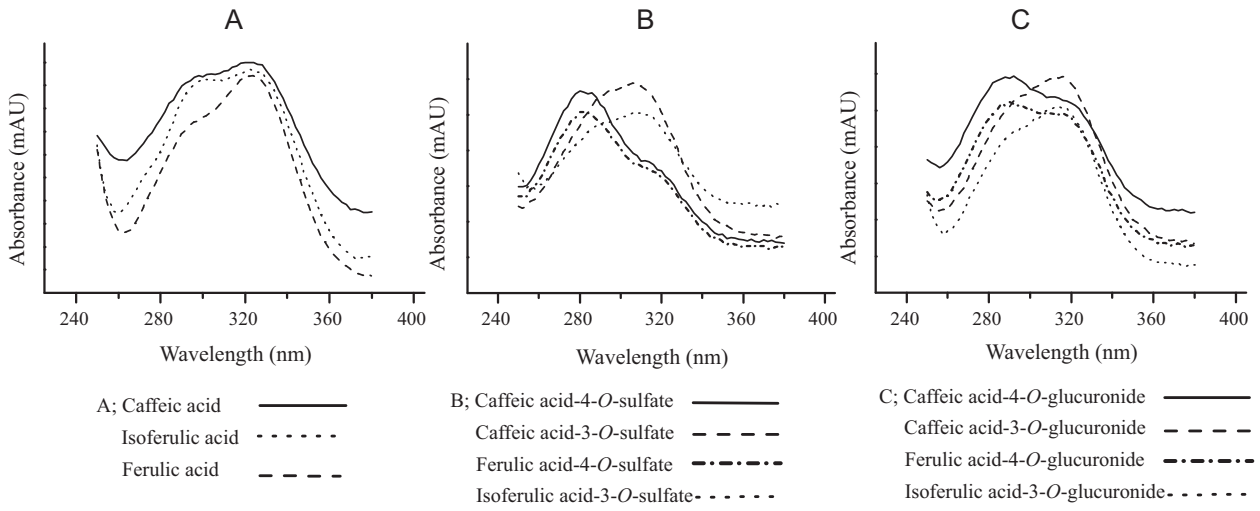


Fig. 3. UV absorbance spectra of hydroxycinnamic acids and conjugates.

3.5. Conjugate response factors on HPLC–DAD and HPLC–DAD–MS^2

The response factors of the sulfate and glucuronide authentic standards were investigated by HPLC–DAD and HPLC–DAD–MS^2 in MRM mode and are summarized in Table 3. For the majority of conjugate standards, the response relative to parent aglycone ranged between 0.6 and 1.2 on HPLC–DAD. However, sulfates of dihydroferulic acid (peak 10), ferulic acid (peak 12) and isoferulic acid (peak 13) respond poorly, possibly attributed to peak broadening during chromatographic elution, whilst caffeic acid-4-*O*-glucuronide (peak 1) response is 1.8 times the response of the parent aglycone on HPLC–DAD. All of the variations are highly dependent on wavelength and were measured at the determined maximum wavelength of each compound (Table 3). In comparison, the relative response of conjugates on HPLC–DAD–MS^2 in MRM mode demonstrated greater variation from that of the aglycone, which is normalized to 1, perhaps as a result of improved ionization efficiency of the conjugates attributed to the presence of the glucuronide or sulfate moiety. Notably sulfated dihydrocaffeic acid (peaks 2 and 3), ferulic acid (peak 12), dihydroferulic acid (peak 10) and isoferulic acid (peak 13) demonstrated a relative response between 3 and 10 times greater than the parent aglycone. This implies that estimations based on the aglycone are inaccurate and it should be noted that response may also be dependent on HPLC–DAD–MS methodology.

3.6. Log *D* determinations

We found that the 9 HCAs had similar Log *D* values; therefore acetylsalicylic acid, alprenolol, atenolol, hydrocortisone and metaprolol were selected in order to allow comparison of experimental and *in silico* values for compounds over a broader range. The experimental apparent partition coefficients (Log *D*) of the selected compounds were compared to *in silico* values (Table 4). Log *D* values were determined at pH 7.4 using a modified shake flask method and the pH of the solutions was constant over 1 h. The results indicate a good correlation (R^2 0.95) between experimental and *in silico* predictions (Fig. 4). The amount of compound required to perform an experimental Log *D* for the glucuronides exceeded supply since the compounds are so water-soluble, and as a result the Log *D* values for these were calculated; this is supported by the excellent correlation between experimental and *in silico* measurements (Fig. 4). The experimental Log *D* values for the sulfates in the organic phase were below the LOD for the HPLC–DAD despite using a high starting concentration of 2 mM, due to the small percentage of uncharged acid at pH 7.4 available to partition into the *n*-octanol phase. Glucuronide and sulfate conjugates were more hydrophilic than the corresponding HCA with increasing hydrophilicity in the order HCAs < sulfates < glucuronides. It was of interest to compare the R_t on reverse-phase chromatography of the conjugates with the Log *D*, as a measure of lipophilicity, at the pH of the analytical solvent to determine the extent of effect of lipophilicity on elution behavior. However, no correlation of conjugate R_t with lipophilicity could be observed (R^2 = 0.003, data not shown), despite a good correlation between R_t of aglycones and lipophilicity (R^2 = 0.94, data not shown).

3.7. Metabolism of ferulic acid by Caco-2 monolayers

All cell culture experiments were performed to examine the apical to basal transport of the free HCA aglycone by Caco-2 cell differentiated monolayers at different apical pH values over 1 h, and samples were analyzed after deproteination without any sulfatase or β-glucuronidase treatment. Cultures incubated with

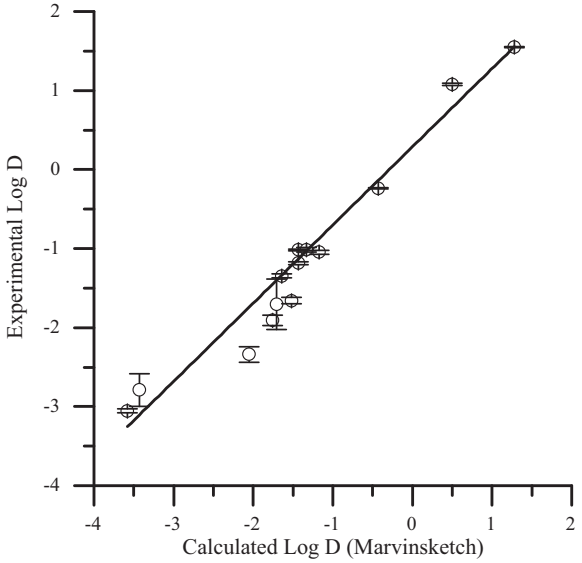


Fig. 4. Comparison of experimental Log *D* and *in silico* calculated Log *D*.

Table 3
HPLC–DAD–MS^2 optimization and characterization of *O*-glucuronides and *O*-sulfate of caffeoyl and feruloyl hydroxycinnamic acids.

Peak[a]	R_t (min)	Wavelength (nm)	Relative peak height (Max Wavelength)	Relative peak height (MRM Mode)	LOQ (nM)	MS optimized parameters		$[M-H]^-$ (*m*/*z*)	MS^2 (*m*/*z*)	Compound
						Fragmentor	Collision energy			
1	6.6	290	1.8	1.0	9	120	20	355	179, 135	Caffeic acid-4-*O*-glucuronide
2	9.8	280	0.8	10.7	5	120	15	261	181, 137	Dihydrocaffeic acid-4-*O*-sulfate
3	10.7	280	0.8	10.3	5	60	15	261	181, 137	Dihydrocaffeic acid-3-*O*-sulfate
4	11.1	280	1.1	2.2	15	120	15	357	181, 137, 113, 175	Dihydrocaffeic acid-4-*O*-glucuronide
5	11.3	290	1.0	1.8	9	90	10	369	193, 113	Ferulic acid-4-*O*-glucuronide
6	11.8	280	0.9	2.5	5	90	10	259	179, 135	Caffeic acid-4-*O*-sulfate
7	13.2	280	0.9	1.8	15	120	20	357	181, 137, 113, 175	Dihydrocaffeic acid-3-*O*-glucuronide
8	13.7	310	1.2	0.5	30	120	20	355	179, 135	Caffeic acid-3-*O*-glucuronide
9	14.6	310	0.8	1.9	5	90	10	259	179, 135	Caffeic acid-3-*O*-sulfate
10	16.4	280	0.4	3.2	3	90	15	275	195, 136	Dihydroferulic acid-4-*O*-sulfate
11	19.6	280	0.3	0.4	50	120	20	371	195, 113	Dihydroferulic acid-4-*O*-glucuronide
12	20.6	280	0.5	5.6	3	90	10	273	193, 178, 134	Ferulic acid-4-*O*-sulfate
13	27	310	0.5	9.8	3	120	15	273	193, 178, 134	Isoferulic acid-3-*O*-sulfate
14	27.1	310	0.6	2.4	9	120	15	369	193, 113, 178	Isoferulic acid-3-*O*-glucuronide
	12.5	280	1	1	50	60	10	181	137.0	Dihydrocaffeic acid
	15.7	320	1	1	15	90	10	179	135.0	Caffeic acid
	31.1	280	1	1	30	90	15	195	136, 121	Dihydroferulic acid
	33.4	320	1	1	68	90	10	193	134, 178, 149	Ferulic acid
	35.8	320	1	1	167	90	10	193	134, 178, 149	Isoferulic acid

[a] For HPLC–DAD–MS^2 chromatogram refer to Figs. 1 and 2. $[M-H]^-$: negatively charged molecular ion. MS^2; product ions produced by fragmentation of $[M-H]^-$, note the first fragment listed is the major product ion and is assigned as the base peak.

ferulic acid released four ferulic acid metabolites (Table 5); dihydroferulic acid (*m*/*z* 195), ferulic acid-4-*O*-glucuronide (*m*/*z* 369), ferulic acid-4-*O*-sulfate (*m*/*z* 273) and dihydroferulic acid-4-*O*-sulfate (*m*/*z* 275). For the first time, unequivocal identification and quantification using authentic standards has confirmed ferulic acid-4-*O*-sulfate and dihydroferulic acid-4-*O*-sulfate as Caco-2 cell metabolites of ferulic acid. Dihydroferulic acid was the most abundant metabolite. Similar equivalent quantities were found in the apical and basal chambers regardless of the apical pH; however, the actual amount in the receiver chamber was 2–3 times greater at pH 6/7.4 (3.97 ± 0.38 pmol/(cm^2 min)) compared to pH 7.4/7.4 (1.17 ± 0.1 pmol/(cm^2 min)). Ferulic acid-4-*O*-sulfate occurred in smaller quantities; apical efflux was observed, but the apical amount was typically lower than that present in the basal chamber. The presence of a proton gradient, pH 6/7.4, favored production of sulfates compared to pH 7.4/7.4 with basal metabolite production values of 1.65 ± 0.16 pmol/(cm^2 min) and 0.95 ± 0.12 pmol/(cm^2 min) respectively. Ferulic acid-4-*O*-glucuronide was present in trace amounts, but higher levels were present basally at apical pH 6/7.4. Dihydroferulic acid-4-*O*-sulfate was detected in the basal chamber below the limit of quantification at pH 6/7.4. In general, the presence of higher proton concentrations (pH 6) promoted the formation and basal release of the ferulic acid metabolites.

3.8. Metabolism of caffeic acid by Caco-2 monolayers

Caffeic acid was transported and analyzed as described above for ferulic acid. Cultures incubated with caffeic acid released 4 metabolites (Table 6); ferulic acid (*m*/*z* 193), caffeic acid-4-*O*-sulfate (*m*/*z* 259), caffeic acid-3-*O*-sulfate (*m*/*z* 259), and ferulic acid-4-*O*-sulfate (*m*/*z* 273). Ferulic acid was the main metabolite and similar amounts of ferulic acid were observed in the apical and basal chambers, with a slightly higher production in the basal chamber in the presence of a pH gradient (pH 6/7.4). For the first time caffeic acid-4-*O*-sulfate and caffeic acid-3-*O*-sulfate were unequivocally identified as caffeic acid metabolites in the intestinal model. Equivalent amounts of caffeic acid sulfates were observed in apical and basal chambers, with higher production at acidic pH 6/7.4. Furthermore, preferential production of caffeic acid-3-*O*-sulfate (0.26 ± 0.01 pmol/(cm^2 min)) compared to caffeic acid-4-*O*-sulfate (0.07 ± 0.01 pmol/(cm^2 min)) was observed. Trace amounts, below the limit of quantification, of ferulic-4-*O*-sulfate were identified as a minor metabolite produced as a further phase II metabolism step.

4. Discussion

HPLC–MS^n has been used previously to identify single position conjugates of HCAs in Caco-2 and human studies. However, to the author's knowledge this is the first time that authentic standards have been used to create mass spectrometry parameters for optimal quantification of individual isomeric HCA conjugates. Analytical validation of the method demonstrated good precision (R.S.D. < 6%) and accuracy (R.E. ± 10%), which is comparable to similar LC–MS methods for quantification of HCA [14] and has excellent sensitivity in the low nmol/l ranges which is up to 15 times more sensitive than previously reported methods [7]. A great advantage of coupling HPLC–DAD to MS^2 is the ability to identify the exact form of metabolites. The unique fragmentation pattern of synthetic standard molecular ions to MS^n product ions is in agreement with the limited number of studies that have identified some HCA metabolites in circulating fluids by LC–MS^n [7,23]. The response factors for HPLC–DAD–MS^2 presented here for the first time are an indication of the ratio of the signal produced by a known amount

Table 4
Comparison of experimental Log *D* and the *in silico* calculated Log *D* and dissociation constant values.

Compound	Experimental Log *D* (pH 7.4)[a]	Calculated Log *D* (pH 7.4)[b]	p*Ka*$_1$ (25 °C)[b]	Ref.
Caffeic acid-4-*O*-glucuronide		−6.99	2.97	
Caffeic acid-3-*O*-glucuronide		−6.99	3.01	
Ferulic acid-4-*O*-glucuronide		−6.79	3.05	
Isoferulic acid-3-*O*-glucuronide		−6.80	3.09	
Dihydrocaffeic acid-4-*O*-glucuronide		−7.08	2.96	
Dihydrocaffeic acid-3-*O*-glucuronide		−7.08	3.01	
Dihydroferulic acid-4-*O*-glucuronide		−6.89	3.05	
Caffeic acid-4-*O*-sulfate	<−4	−3.98	−2.35	
Caffeic acid-3-*O*-sulfate	<−4	−3.98	−2.30	
Ferulic acid-4-*O*-sulfate	<−4	−4.46	−2.25	
Isoferulic acid-3-*O*-sulfate	<−4	−4.46	−2.20	
Dihydrocaffeic acid-4-*O*-sulfate	<−3.5	−4.07	−2.28	
Dihydrocaffeic acid-3-*O*-sulfate	<−3.5	−4.07	−2.23	
Dihydroferulic acid-4-*O*-sulfate	<−3	−4.55	−2.18	
Caffeic acid	−1.35	−1.64 (−166)	3.84 (4.43, 4.36)	[27–29]
Ferulic acid	−1.19	−1.43 (−1.24)	3.97 (4.44)	[26,29]
Isoferulic acid	−1.02	−1.43	3.97	
Dihydrocaffeic acid	−1.70	−1.71 (−2.26)	3.84 (4.51, 4.36)	[26,27,29]
Dihydroferulic acid	−1.66	−1.54	3.95 (4.59)	[26]

( ): Experimental p*Ka*$_1$ and Log *D* values taken from literature. Note: dissociation constant (p*Ka*) at half neutralization. <: Log *D* values below LOQ.
[a] Experimental Log *D* determined by octanol:water partition at 37 °C. The average recovery of each aglycone from partition phases was between 80 and 100% ($n = 5$).
[b] Calculated Log *D* and p*Ka* values taken from [22].

Table 5
Metabolism of ferulic acid by differentiated Caco-2 monolayers ($n = 3$).

Metabolite	pH gradient	Metabolite production			
		Apical		Basal	
		pmol/(cm^2 min)	% of apical dose	pmol/(cm^2 min)	% of apical dose
Ferulic acid	6/7.4	2316 ± 410	(65)	770 ± 77	(22)
	7.4/7.4	2522 ± 91	(73)	290 ± 26	(8)
Dihydroferulic acid	6/7.4	2.85 ± 0.20	(0.08)	3.97 ± 0.38	(0.11)
	7.4/7.4	1.11 ± 0.04	(0.03)	1.17 ± 0.10	(0.03)
Ferulic acid-4-*O*-sulfate	6/7.4	0.75 ± 0.13	(0.02)	1.65 ± 0.16	(0.05)
	7.4/7.4	0.64 ± 0.04	(0.02)	0.95 ± 0.12	(0.03)
Ferulic acid-4-*O*-glucuronide	6/7.4	<0.08		0.24 ± 0.07	(0.01)
	7.4/7.4	n.d		<0.08	
Dihydroferulic acid-4-*O*-sulfate	6/7.4	n.d		<0.02	
	7.4/7.4	n.d		n.d	

Cultures were incubated (1 h) in the presence of ferulic acid (500 μM). Apical pH adjusted to 6 or 7.4; basal pH 7.4. Sample analysis performed on LC–MS^n, metabolites confirmed by product ion in MRM mode: dihydroferulic acid (*m*/*z* 195), ferulic acid-4-*O*-glucuronide (*m*/*z* 369), ferulic acid-4-*O*-sulfate (*m*/*z* 273), dihydroferulic acid-4-*O*-sulfate (*m*/*z* 275). <: Below the limit of quantification; n.d: not detected. Values are mean ± SD ($n = 3$). ( ): Percentage of the initial amount of ferulic acid added to the apical chamber.

of analyte and may be used as essential correction factors when quantifying HCA conjugates in the native intact form and using parent aglycone calibration curves. We observed that quantification calculated relative to the aglycone by HPLC–DAD was more accurate than HPLC–DAD–MS^2, although of course less sensitive. The observed increase in HPLC–MS signal for the conjugate relative to the corresponding aglycone may be attributed to ionization suppression of the aglycone. Mechanistic investigation of ioniza-

Table 6
Metabolism of caffeic acid by differentiated Caco-2 monolayers ($n = 3$).

Metabolite	pH Gradient	Metabolite Production			
		Apical		Basal	
		pmol/(cm^2 min)	% of apical dose	pmol/(cm^2 min)	% of apical dose
Caffeic acid	6/7.4	3447 ± 111	(96)	138 ± 16	(4)
	7.4/7.4	3242 ± 6	(91)	68 ± 6	(2)
Ferulic acid	6/7.4	0.90 ± 0.25	(0.03)	0.85 ± 0.05	(0.02)
	7.4/7.4	0.94 ± 0.07	(0.03)	0.68 ± 0.02	(0.02)
Caffeic acid-3-*O*-sulfate	6/7.4	0.26 ± 0.03	(0.01)	0.26 ± 0.01	(0.01)
	7.4/7.4	0.09 ± 0.01	(0.003)	0.09 ± 0.01	(0.003)
Caffeic acid-4-*O*-sulfate	6/7.4	0.06 ± 0.01	(0.002)	0.07 ± 0.01	(0.002)
	7.4/7.4	<0.03		<0.03	
Ferulic acid-4-*O*-sulfate	6/7.4	<0.02		<0.02	
	7.4/7.4	n.d		<0.02	

Cultures were incubated (1 h) in the presence of caffeic acid (500 μM). Apical pH adjusted to 6 or 7.4; basal pH 7.4. Sample analysis performed on LC–MS^n, metabolites confirmed by product ion in MRM mode: caffeic acid-3-*O*-sulfate (*m*/*z* 259), caffeic acid-4-*O*-sulfate (*m*/*z* 259), ferulic acid (*m*/*z* 193), ferulic acid-4-*O*-sulfate (*m*/*z* 273). <: Below the limit of quantification. n.d: not detected. Values are mean ± SD ($n = 3$). ( ): Percentage of the initial amount of caffeic acid added to the apical chamber.

tion in ESI mode has been reported previously [24] and point to interference by non-volatile solutes leading to loss of net charge of the analyte as the main cause of ionization suppression in biological samples. In the current study, interference due to the sample matrix is unlikely because response factor was determined with samples prepared in high purity (LC–MS grade) solvents. However, the poor sensitivity of aglycones compared to their conjugate analogs may be explained by the acid content of the mobile phase. Particular solvent system additives conventionally used to aid ionization or improve chromatography such as acetic acid or formic acid are known to suppress certain carboxylic acid analytes [25] by formation of acid hetero-dimers. It may be postulated that conjugation of the aglycone disrupts the formation of adducts leading to increased abundance of the molecular ion and hence improved sensitivity for conjugates compared to aglycones, although the molecular modeling of this interaction is beyond the scope of this paper. Alternatively, the higher sensitivity of the conjugates compared to their aglycone analog may be attributed, in part, to improved ionization of the conjugate due to the fact the glucuronic acid and sulfate are more acidic than carboxylic acid, as predicted by their respective p*K*a values (Table 4). Therefore, conjugated HCAs will form a charge more efficiently in ESI than the less acidic HCA aglycones. In the absence of authentic metabolite standards, the practice of quantification by HPLC–MS as aglycone equivalents should be discouraged as this can lead to an under- or over-estimation of the extent of metabolism.

Interestingly, UV absorbance spectra (Fig. 3) determined by HPLC–DAD may provide a useful secondary method for distinguishing between isomeric forms of HCA metabolites if there is a sufficiently high sample concentration. In the present study, a distinct shift in absorbance maxima to a shorter wavelength was observed when HCAs are conjugated in the *para* (4-*O*-) position when compared to conjugation in the *meta* (3-*O*-) position. This is potentially explained by the shorter structural continuity of single and double bonds or lone pair electrons e.g. oxygen in the ester bond, created by *para* position conjugation that leads to higher orbital energy and hence absorbance at shorter wavelength [18]. When conjugated in the *meta* position the continuity of bonding is extended by an additional C=C bond, which increases the length of conjugation producing a chromophore with absorbance at a wavelength similar to that of the parent aglycone. This spectral property may be useful for HPLC–DAD distinction between isomers of additional HCA metabolites in the absence of authentic standards.

The lipophilicity of HCAs and metabolites was also investigated and we demonstrated, as expected, that conjugation either by sulfation or glucuronidation significantly increased hydrophilicity (Table 4). Linear regression of the selected compounds indicated a good correlation (R^2 0.95) between experimental and *in silico* predictions (Fig. 4). Calculated Log *D* values were found to be a reliable estimate of lipophilicity for aglycone HCAs compared to our data and are in line with those calculated from Log *P* and p*K*a values in literature [26–30] and were used for prediction of conjugate Log *D* in the absence of sufficient amounts of standard for detection by HPLC–DAD. For the first time, we present physicochemical estimates of lipophilicity for HCA sulfate and glucuronide metabolites. Previously, R_t on reverse-phase chromatography of a series of phenolic aglycones were compared with lipophilicity [12], and showed agreement with our observation that elution time of the aglycone is strongly correlated with Log *D* at the pH (2.2) of the analytical solvents ($R^2 = 0.94$, data not shown). However, it was also shown in this current study that elution time of the phenolic acid conjugates on reverse-phase chromatography showed no correlation with lipophilicity ($R^2 = 0.003$, data not shown). It is apparent that lipophilicity has less influence on the chromatographic behavior for conjugates than for the corresponding aglycones. These results indicate that the greater structural complexity and ionized nature at pH 2.2 (glucuronides, p*K*a ~ 3.0 have approximately 15% of the molecules in the ionized form, but the sulfates, p*K*a ~ 2.2 are 100% ionized because of the sulfate moiety) of the conjugates plays a stronger role in their physicochemical interactions with the bonded column phase than lipophilicity. Thus, our data indicates that previous methods based on reverse phase chromatography are an unreliable measure of conjugate lipophilicity. An estimate of relative lipophilicity for free hydroxycinnamic acid, sulfated, and glucuronidated conjugates is provided for the first time and are ranked in increasing lipophilicity: glucuronides < sulfates ≪ HCAs Furthermore, the differing lipophilicities between sulfate and glucuronide conjugates may influence their membrane and transporter interactions. For example, OAT1, an organic anion transporter expressed in human kidney cells, has greater affinity for sulfated HCA than those with glucuronide conjugation [10], which may contribute to increased excretion of sulfated HCAs compared to glucuronides in human urine [31]. Ultimately the mode of metabolism is a major factor determining bioavailability *in vivo*.

In the present study, ferulic acid and caffeic acid were incubated with Caco-2 intestinal cells to investigate the pharmacokinetic fate and nature of metabolites released following HCA absorption and metabolism. HPLC–DAD–MS^2 analysis of released conjugates, compared to 14 synthetic standards, allowed unequivocal identification of positional isomers and reliable quantification of conjugate bioavailability. In total, 4 metabolites were identified following incubation of ferulic acid with Caco-2 monolayers: dihydroferulic acid, ferulic acid-4-*O*-sulfate, ferulic acid-4-*O*-glucuronide and dihydroferulic acid-4-*O*-sulfate (Table 5). The reduced form of ferulic acid was the predominant metabolite, suggesting that intestinal enterocytes show a preferential metabolism towards reductase activity. Furthermore, ferulic acid-4-*O*-sulfate was the main conjugate present basally, with small amounts present as dihydroferulic acid-*O*-sulfate. This data is in agreement with *in vitro* data which indicated that sulfotransferase SULT1E1 has a unique regio-selectivity for catalyzing sulfation of the 4-hydroxyl of ferulic acid and dihydroferulic acid with high efficiency [31]. Trace amounts of glucuronidation were observed, most likely produced by UDP-glucuronosyltransferase 1A1 [31].

Notably *in vitro* and *in vivo* metabolism studies also observed that sulfate conjugates occur in greater amounts in urine than their glucuronide equivalents [31]. Metabolism of ferulic acid by Caco-2 cells has been studied previously, where ferulic acid was exported as ferulic acid-4-*O*-sulfate [15] and more recently as dihydroferulic acid and ferulic acid-4-*O*-glucuronide by Caco-2/HT29 MTX co-cultures [12], which was confirmed by HPLC–MS^n and only a limited number of available standards. In the latter study, no ferulic acid glucuronide could be detected in Caco-2 cell monocultures [12]. Neither ferulic acid-4-*O*-glucuronide nor dihydroferulic acid-4-*O*-sulfate have been identified as ferulic acid metabolites in Caco-2 intestinal cells before. Our results are consistent with the observations of a recent human study showing rapid appearance of ferulic acid-4-*O*-sulfate, dihydroferulic acid-4-*O*-sulfate and dihydroferulic acid, but no ferulic acid-*O*-glucuronide could be detected in 0–2 h urine following coffee consumption [7].

Metabolism of caffeic acid in the present study indicated that methylation, most probably by cytosolic COMT, is the predominant metabolic pathway. The methylated caffeic acid is released as ferulic acid; since no isoferulic acid was detected it is likely that COMT exhibits highest activity for methylation of the *meta* hydroxyl on the phenol ring. Similar amounts of methylated caffeic acid were distributed in apical and basal chambers, with a slight enhancement of production in the basal chamber in the presence of a proton gradient (pH 6/7.4). Previously ferulic acid, mainly in the free form, was observed following *in situ* perfusion of caffeic acid in the small intestine of rats [32], accounting for 0.5% of

the perfusate, which is similar in magnitude to our current study (approximately 0.1%). Ferulic acid, quantified as the free form after enzymatic hydrolysis, was also observed in plasma of rats after ingestion of caffeic acid [33]. Furthermore, human studies indicate ferulic acid metabolites appear in plasma and urine within 2 h following coffee consumption [7], possibly due to FQA cleavage to ferulic acid by intestinal microflora. Our current data advance our knowledge of CQA metabolism by highlighting the potential for intestinal methylation of caffeic acid after liberation from CQA, which may contribute to the pool of feruloyl derivatives. A smaller amount of caffeic acid was sulfated by intestinal cells; we have for the first time unequivocal identification and quantification of caffeic acid-3-*O*-sulfate and caffeic acid-4-*O*-sulfate metabolites. Confirmation of positional substitution was previously an analytical challenge and consequently indirect quantification of caffeic acid sulfate in rat plasma was achieved post-enzymatic hydrolysis [33]. Regio-specificity of caffeic acid sulfation by most likely SULT1A1, appears to have a 4 times higher affinity for sulfation of the *meta* hydroxyl of caffeic acid, leading to preferential release of caffeic acid-3-*O*-sulfate (0.26 ± 0.01 pmol/(cm^2 min)) compared to caffeic acid-4-*O*-sulfate (0.07 ± 0.01 pmol/(cm^2 min)) at pH 6/7.4. Our data are in agreement with recent *in vitro* enzyme kinetic and human studies, which indicated higher amounts of caffeic acid-3-*O*-sulfate (1.8 μM) than caffeic acid-4-*O*-sulfate (0.2 μM) in 2 h urine [7] and that SULT1A1 has a high activity towards caffeic acid with a specificity for 3-hydroxyl of the catechol group [31]. Trace amounts of ferulic acid-4-*O*-sulfate were identified, suggesting the capacity of intestinal cells for multiple steps of phase II metabolism involving COMT and SULT.

In conclusion, for the first time novel physicochemical properties of authentic HCA conjugates have been characterized. Data resulting from our characterization will permit improved accuracy of HCA conjugate quantification even in the absence of authentic standards. To the author's knowledge, this is the first method to develop MS operating conditions optimized for individual HCA conjugates, and the method allowed four novel metabolites of monoculture Caco-2 metabolism to be unequivocally identified and quantified.

Acknowledgement

We thank Nestle Research Center, Switzerland, for funding, including a studentship to TF.

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