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Investigation of the metabolic fate of dihydrocaffeic acid

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ABSTRACT

The antioxidant dihydrocaffeic acid is a dietary constituent and a microbial metabolite of flavonoids. Orally administered to rats, dihydrocaffeic acid was very rapidly absorbed most probably by the gastric or duodenal epithelium and excreted in urine as free and conjugated forms. LC–MS^2 analysis of plasma and urine samples allowed confident identification of the dihydrocaffeic acid metabolites. The parent compound was glucuronidated, sulphated or methylated, on one of the hydroxyl groups present on its phenyl ring. All the dihydrocaffeic acid metabolites peaked in plasma within the first 30 min following ingestion, suggesting a metabolism possibly by the gastric or duodenal cells and by the liver. Using *in vitro* and *ex vivo* models of the intestinal epithelium and the liver, the identity and source of the metabolites detected *in vivo* were examined. The data obtained suggest that, in rats, intestinal cells are more able to glucuronidate dihydrocaffeic acid, whereas liver favours sulphation. Moreover, glucuronidation, sulphation and methylation seem to be regio-selective, preferably on the 3-OH of dihydrocaffeic acid. The methyl conjugate, dihydro-ferulic acid, was shown to be oxidized into ferulic acid by intestinal and hepatic cells, which were also able to perform the reverse reaction, the reduction of ferulic acid into dihydro-ferulic acid. As a conclusion, the main form of dihydrocaffeic acid circulating in plasma after its ingestion is a mixture of different primary and secondary metabolites.

1. Introduction

Hydroxycinnamic acids, which derive from cinnamic acid, are abundantly found in edible plants [1]. Except in processed food where they can be found as free forms, they are usually present as simple esters with quinic acid, glucose, polysaccharides or other carboxylic acids such as tartaric or shikimic acids [2]. Even though not one of the most commonly found hydroxycinnamic acids [3], dihydrocaffeic acid is present in various foods. It is one of the major phenolic antioxidants abundantly present in black olive pericarp, and in lower amounts in brined black olive, with traces in brined green olives [4]. It has also been detected in Mongolian medicinal plants as feruloylpodospermic acid, which is composed of two dihydrocaffeic acid units esterified at position 1 and 5 of the quinic acid moiety, with an additional feruloyl group being attached to position 3 [5]. Moreover, dihydrocaffeic acid is present in blood and in urine as a metabolite of various polyphenols found in food, beverages, medicinal plants or extracts. It was detected in the plasma of coffee drinkers [6], in urine as the free form and mainly conjugated in human plasma after ingestion of artichoke leaf extracts which contain monocaffeoyl-quinic, dicaffeoyl-quinic and caffeic acids as well as flavonoids [7], in human urine after chocolate intake [8]

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Abbreviations: LC–MS, liquid chromatograph with mass spectrometry; MRM, multiple reaction monitoring; *m/z*, mass to charge ratio; TIC, total in count.

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and in rat urine after ingestion of polyphenol rich red wine extract [9]. Moreover, dihydrocaffeic acid, one of the major phenolic acids found in human fecal water [10], is expected to be mainly released from its precursors after microbial degradation. It results from the microbial hydrogenation of the side chain of caffeic acid [11] and from the microbial degradation of catechin [12] and procyanidins [13,14]. It is probable that microbial cinnamoyl esterases would be able to release dihydrocaffeic acid from feruloylpodospermic acid or any ester of dihydrocaffeic acid present in food, as has been proposed for ferulic acid [15]. Since caecum and colon contain the highest amount of bacteria of the gastrointestinal tract [16], dihydrocaffeic acid is mainly expected to be released in this region of the intestine.

As for many other hydroxycinnamic acids, dihydrocaffeic acid exerts potent antioxidant properties. Towards the stable free radical DPPH$^{\bullet}$, dihydrocaffeic acid had a higher scavenging effect than (±)-α-tocopherol [17], and was even more efficient than caffeic acid [18]. Using a FRAP assay, its antioxidant power was shown to be similar to caffeic and 5-caffeolylquinic acid in PBS and in plasma [19]. Unlike 5-caffeolylquinic acid, dihydrocaffeic acid could also enter erythrocytes and enhance their ability to reduce the transmembrane oxidant stress generated by extracellular ferricyanide. Cellular incorporation of dihydrocaffeic acid was also demonstrated with endothelial cells, which were consequently protected against oxidative stress and exhibited slightly increased eNOS activity [20]. As a microbial metabolite of catechins and procyanidins, dihydrocaffeic acid could conceivably be partly responsible for some biological effects observed after consumption of green tea or chocolate [21,22].

However, the potential biological activity of dihydrocaffeic acid depends on its absorption and further metabolism. Thus, the present work was performed in order to better understand the absorption and metabolism of dihydrocaffeic acid, firstly by identifying the main metabolites resulting from its ingestion and secondly by determining the sites of production of these metabolites. The first site of metabolism investigated was the epithelium of the colon, where dihydrocaffeic acid is more likely to be absorbed. Since the second detoxifying organ encountered by xenobiotics is the liver, we also tested its capacity to metabolize dihydrocaffeic acid.

2. Materials and methods

2.1. Materials

Dulbecco's Modified Eagle's medium (DMEM), RPMI 1640, fetal calf serum (FCS), penicillin–streptomycin, amphotericin B, L-glutamine, L-ascorbic acid, Hanks' balanced salt solution (HBSS), Earle's balanced salt solution (EBSS), methionine, insulin, hydrocortisone 21-hemisuccinate, Krebs–Ringer bicarbonate buffer, sodium bicarbonate, calcium chloride, D-glucose and fluorescein isothiocyanate-dextran 4000 (FD4) were purchased from Sigma (Buchs, Switzerland). Minimum essential medium (MEM) and gentamicin were purchased from Gibco (Basel, Switzerland). Plastic dishes and Transwell® Polycarbonate semi-permeable membranes of 0.4 μm pore size and 4.7 cm^2 surface area were obtained from Corning (Wohlen, Switzerland). Ethyl acetate, sodium acetate anhydrous, HPLC grade water, acetonitrile, formic and glacial acetic acids were purchased from Merck (Dietikon, Switzerland). Isoferulic, ferulic, caffeic and dihydrocaffeic acids were obtained from Extrasynthese (Genay, France). Dihydroferulic acid was provided by Alfa Aesar (Karlsruhe, Germany). Dihydrocaffeoyl-3-O-β-D-glucuronide and dihydrocaffeoyl-4-O-β-D-glucuronide, as HPLC standards, were from the Nestlé Research Center collection of standard compounds.

2.2. Cell culture

The human colon carcinoma cell line Caco-2 cells (HTB-37) was obtained from the American Type Culture Collection (ATCC, LGC Promochem, Molsheim, France). The HT29-MTX cells were elaborated by Dr T. Lesuffleur (Villejuif, France). Caco-2 and HT29-MTX cells were used between p 30 and 70. The cells were maintained in 75 cm^2 culture flasks at 37 °C under a humidified 5% CO_2/95% O_2 atmosphere in DMEM supplemented with 584 mg/l of L-glutamine, 1% (v/v) MEM, 100 U/ml of penicillin–streptomycin, 0.25 μg/ml amphotericin B and 15 or 10% heat inactivated FCS, respectively, for Caco-2 or HT29-MTX.

2.3. Metabolism experiments

HT29-MTX or Caco-2 were seeded at 6×10^4 cells/cm^2 in 3.8 cm^2 wells and grown over a period of 6 days in 10% FCS supplemented DMEM, the medium being changed every second day. Metabolism was studied by incubating each well with 100 μM dihydrocaffeic acid in a volume of 1.5 ml. After 3 h of incubation at 37 °C, supernatant was collected, acidified with acetic acid to obtain a final concentration of 10 mM and stored at −20 °C until analysis.

2.4. In vitro transepithelial transport studies

Caco-2 and HT29-MTX were seeded together in Transwells at 6×10^4 cells/cm^2 (76% Caco-2 and 24% HT29-MTX). Co-cultures were allowed to grow and differentiate over a period of 21 days, the medium (DMEM containing 10% FCS) being changed every other day. The integrity of the monolayers was evaluated by measurement of the transepithelial electrical resistance (TEER) using a Millicell®-ERS device (Millipore, Zug, Switzerland) before and after the treatments. To evaluate transepithelial permeability, medium was removed from the apical and basal chambers and replaced by 2 ml of the transport solution consisting of HBSS containing 1.8 mM calcium and dihydrocaffeic acid (apical side) at 1 mM, the pH being adjusted to 6 or 7.4. After incubation at 37 °C, apical and basal solutions were collected, acetic acid added to obtain a final concentration of 10 mM and samples stored at −20 °C until further analysis.

2.5. Animals for the ex vivo studies

Male Sprague–Dawley rats (6-week old) were adapted to the laboratory conditions (normal 12 h dark/12 h light cycles) with free access to tap water and to a conventional diet for 1 week, and then to a polyphenol-free semi-synthetic diet (AIN 93M

type) for another week. Rats were sacrificed randomly by decapitation.

2.6. Transport study using everted sacs

A part of the jejunum as well as the ascending and the descending colon were quickly excised after the sacrifice, everted and rinsed in room temperature Krebs–Ringer bicarbonate buffer, prepared according to the manufacturer's recommendations, supplemented with 1.8 mM calcium, and gassed with 95% O_2/5% CO_2 for 60 min prior to use. The segments were then mounted at one extremity on a 1 ml plastic syringe which contained 0.7 ml of Krebs–Ringer bicarbonate buffer, pre-warmed to 37 °C and supplemented with 1 mg/ml FD4, while the other extremity was sealed with surgical linen. The content of the syringes was emptied into the sacs, which were then incubated in chambers filled with 30 ml of Krebs–Ringer bicarbonate buffer pre-warmed to 37 °C and containing dihydrocaffeic acid (500 μM). The everted sacs were incubated for 30 min in the chambers maintained at 37 °C and continuously gassed with 95% O_2/5% CO_2. At the end of the incubation, the content of the sacs was recovered by filling back the syringe, further acidified with acetic acid to a final concentration of 10 mM and stored at −20 °C until analysis. The content of the chambers was recovered in order to control the integrity of the sacs by measuring the FD4 concentration by fluorescence spectrophotometry released by the sacs (excitation at 490 nm and emission at 520 nm).

2.7. Liver metabolism

After sacrifice, the livers were immediately excised, placed in ice cold EBSS, which had been previously supplemented with 25 mM D-glucose and gassed with 95% O_2/5% CO_2 for 1 h. Cores of liver were prepared using a hand-held coring tool of 8 mm diameter (Vitron, Tucson, USA). Liver slices of 200–250 μm thickness were prepared using a Krumdieck tissue slicer (Alabama Research and Development Corp., Munsford, AL, USA). The slices were preconditioned by incubating them for 30 min into culture medium made of RPMI 1640 supplemented with 5% fetal calf serum, 0.5 mM methionine, 1 μM insulin, 50 μg/ml of gentamicin and 0.1 mM hydrocortisone 21-hemisuccinate and pre-warmed for 16 h at 37 °C under an atmosphere of 5% CO_2/95% air. After adaptation of the liver slices to the culture conditions, they were transferred to preconditioned medium containing 50 μM of dihydrocaffeic acid and 50 μM of ascorbic acid. Liver slices were incubated at 37 °C under an atmosphere of 5% CO_2/95% air for different times. At the end of each incubation time, supernatants of cultures were collected and acidified to a final concentration of 10 mM acetic acid.

2.8. In vivo study of dihydrocaffeic acid metabolism

Eight-week-old male Sprague–Dawley rats were adapted to laboratory conditions (normal 12 h dark/12 h light cycles) with free access to tap water and to a conventional diet for 1 week and then to a polyphenol-free semi-synthetic diet (AIN 93M type) for another week. The rats were adapted to metabolic cages for 48 h and food was withheld 16 h before the experiment. The rats were randomly orally administered, by gastric gavage, either 100 μmol/kg of dihydrocaffeic acid prepared as a 20 mM solution in water or water as control. Four hundred microlitres of blood were collected into heparinized tubes after incision of the tail vein before and at different time points following administration of the treatment (30 min, 1, 2, 4, 6, 8, 10 and 24 h). Plasma was obtained by centrifugation of the blood samples for 5 min at 3000 × *g*. Urine was also collected from the metabolic cages before and 6, 12 and 24 h after administration of dihydrocaffeic acid. Plasma and urine were acidified with acetic acid to a final concentration of 10 mM and stored at −80 °C until analysis.

2.9. Sample preparation and LC–MS analysis for the in vitro and ex vivo studies

Room temperature defrosted samples (150 μl) were mixed with an equal volume of 0.1 mM sodium acetate buffer pH 5. HPLC grade water (150 μl) and 200 mM HCl/methanol (300 μl) were added. After sonication, the mixture was vortexed and extracted three times with ethyl acetate. After each centrifugation for 5 min at 5000 × *g*, organic phases were collected, pooled and dried under nitrogen. The dried extracts were re-suspended into 50 μl 5% acetonitrile in water containing 0.1% formic acid and injected onto an Acquity UPLC™ BEH Shield RP_{18} column (2.1 mm × 100 mm, 1.7 μm; Waters, Rupperswil, Switzerland). For MRM analysis, two ethyl acetate extracts per sample were pooled and re-suspended into 30 μl of solvent. Elution was performed with a flow rate of 0.3 ml/min and a gradient of solvent A (water) and B (acetonitrile), both acidified with 0.1% formic acid. HPLC analysis was started with 3% of solvent B. This condition was maintained for 1 min and then the percentage of solvent B was linearly increased to 5 within 1 min, to 10 within 18 min, to 15 within 10 min and finally to 100 within 1 min. One hundred percent solvent B was maintained for 3 min, then initial conditions were reached within 1 min and the column equilibrated in 3% solvent B for 5 min. The HPLC system (Acquity UPLC system, Waters, Rupperswil, Switzerland) was connected to a triple Quadrupole Micromass Quattro micro API mass spectrometer (Waters, Rupperswil, Switzerland), with an electrospray ionization (ESI) interface. The LC eluate was introduced directly from the absorbance monitor (Photo Diode Array, PDA) into the ESI probe without flow splitting. A nebulizing gas flow of 150 L/h and a drying gas flow of 550 L/h were applied for ionization using nitrogen in both cases. Samples were analyzed using the negative ion mode. ESI-MS parameters were as follows: voltage, 3 kV; capillary temperature, 90 °C. The results obtained for the amount of the phenolic acids were normalized using an internal standard, syringic acid, added at a known concentration to the samples to be extracted.

2.10. Sample preparation and LC–MS analysis for the in vivo study

Room temperature-defrosted plasma (50 μl) or urine (200 μl) was mixed with an equal volume of 0.1 mM sodium acetate buffer pH 5 and of HPLC grade water. Twice the initial volume of sample in 200 mM HCl/methanol was added. After sonication, the mixture was extracted three times with a volume of ethyl acetate corresponding to six times the initial volume of

sample. After each centrifugation at 5000 × *g* for 5 min, organic phases were collected, pooled and dried under nitrogen. Dried extracts were re-suspended into 100 μl 15% acetonitrile in water. The extracts were analysed by reverse-phase HPLC (10 μl) using an Atlantis TM dC18 column (4.6 mm × 100 mm, 3 μm; Waters). Solvent A (5% acetonitrile) and solvent B (100% acetonitrile) were run at a flow rate of 300 μl/min using the following gradient: starting at 100% solvent A, solvent B was increased to 66% in 25 min, to 100% in further 2 min and maintained at 100% for 2 min, returned to initial conditions within 1 min, and maintained for 10 min. The eluent was monitored by UV with a DAD system set up at 280 nm and the metabolites identified by their *m/z* value using an MS/MS detection system (LCQ Deca XP$^+$, Thermofinnigan, Cambridge, UK). The identity of the different compounds was estimated with reference to the ratio mass to charge by general scan (MS^1) and to the daughter ions by fragmentation of the parent ions (MS^2). ESI-MS parameters were as follows: voltage, 5.5 kV; capillary temperature, 365 °C.

2.11. *Data analysis*

Data are shown as mean ± S.D. Differences between test and control conditions were assessed by Student's *t*-test and differences with value of $P < 0.05$ were considered as significant.

3. Results

3.1. *In vivo study of the dihydrocaffeic acid fate*

In order to better understand its absorption and metabolism, dihydrocaffeic acid (structure shown in Fig. 1) was orally administered to rats. The study was designed for the identification of metabolites using a high dose of dihydrocaffeic acid (100 μmol/kg). Blood and urine samples were collected and analyzed by LC–MS using an ion trap for optimal compound identification. The approach was firstly to detect the major peaks by total ion scan of the samples. Then, we looked for specific metabolites predicted to be present in the blood and urine after dihydrocaffeic acid ingestion and for which mass to charge ratio of the respective $[M - H]^-$ ions could be calculated such as the glucuronide (*m/z* 357), sulphate (*m/z* 261), sulpho-glucuronide (*m/z* 437), methyl (*m/z* 195) and glycine (*m/z* 238) conjugates, but also derivatives of dihydrocaffeic acid with an unsaturated or truncated side chain. The identity of the compounds detected was confirmed by studying the MS^2 fragmentation profile and using the corresponding standards if available. Full scan analysis of plasma samples collected after dihydrocaffeic acid administration showed no presence of the $[M - H]^-$ ion corresponding to the ingested compound (*m/z* 181). However, two $[M - H]^-$ ions at *m/z* 357 and two at *m/z* 261 were detected, suggesting that dihydrocaffeic acid was glucuronidated or sulphated on either the 3- or 4-hydroxyl groups of the phenyl ring. Their identity was confirmed by MS^2 fragmentation of the parent ions producing both a $[M - H]^-$ daughter ion at *m/z* 181 corresponding to dihydrocaffeic acid (Fig. 2A and B). Moreover, the $[M - H]^-$ ion at *m/z* 357 produced two additional $[M - H]^-$ ions at *m/z* 175 and 113, respectively identified as the glucuronic acid moiety and its daughter ion $[M - H–H_2O–COOH]^-$. However, neither double sulfate/glucuronide, nor sulfo-glucuronide, of dihydrocaffeic acid was detected in the plasma samples. In addition to conjugation with glucuronide or sulphate, dihydrocaffeic acid was also methylated, but only one peak was detected for this mass to charge ratio (195). The identity of dihydroferulic acid was confirmed by MS^2 analysis, the fragmentation of the $[M - H]^-$ ion at *m/z* 195 producing the main daughter ions $[M - H–COOH]^-$ (*m/z* 151) and $[M - H–CH_3–COOH]^-$ (*m/z* 136), as obtained for authentic dihydroferulic acid. In addition, two $[M - H]^-$ ions at *m/z* 193 were detected in plasma, which upon fragmentation gave three different ions at *m/z* 149, 178 and 134, corresponding respectively to the $[M - H–COOH]^-$, $[M - H–CH_3]^-$ and $[M - H–CH_3–COOH]^-$ daughter ions of 193. The identity of the two compounds at *m/z* 193 was confirmed using standards of ferulic and isoferulic acid. Interestingly, dihydroferulic, ferulic and isoferulic acids were also found as sulphated forms, the respective parent ions at *m/z* 275 and 273 producing daughter ions at *m/z* 195 and 193, the un-conjugated forms, by MS^2 analysis. All the metabolites circulating in plasma reached a maximum within the first 30 min following the oral administration of dihydrocaffeic acid, their peak area decreasing between 30 and 60 min and being completely cleared after 120 min, except for the dihydrocaffeoyl-sulphates. The parent compound dihydrocaffeic acid was present at low level (less than 1% of the total dose) in urine collected 6 h (but not 12 and 24 h) after its oral administration. All the dihydrocaffeic acid metabolites present in plasma (see above) were also detected in urine collected at 6, 12 and 24 h, except for the sulphate and glucuronide conjugates of dihydrocaffeic acid, which were not found at the 24 h time point.

Having identified the potential metabolites circulating in plasma and excreted in urine, dihydrocaffeic acid absorption and metabolism were studied using *ex vivo* and *in vitro* models of colonic epithelium where dihydrocaffeic acid is most likely expected to appear from ingested food. Samples were analysed by LC–MS using a Quadrupole, allowing quantification of the metabolites in addition to their identification.

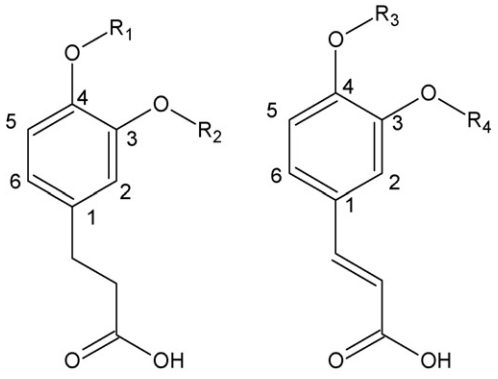

Fig. 1 – Chemical structures of phenolic acids. R1, R2 = H: dihydrocaffeic acid, R1 = H; R2 = CH_3: dihydroferulic acid; R1 = CH_3; R2 = H: dihydroisoferulic acid, R3 = H; R4 = CH_3: ferulic acid; R3 = CH_3; R4 = H: isoferulic acid, R3, R4 = H: caffeic acid.

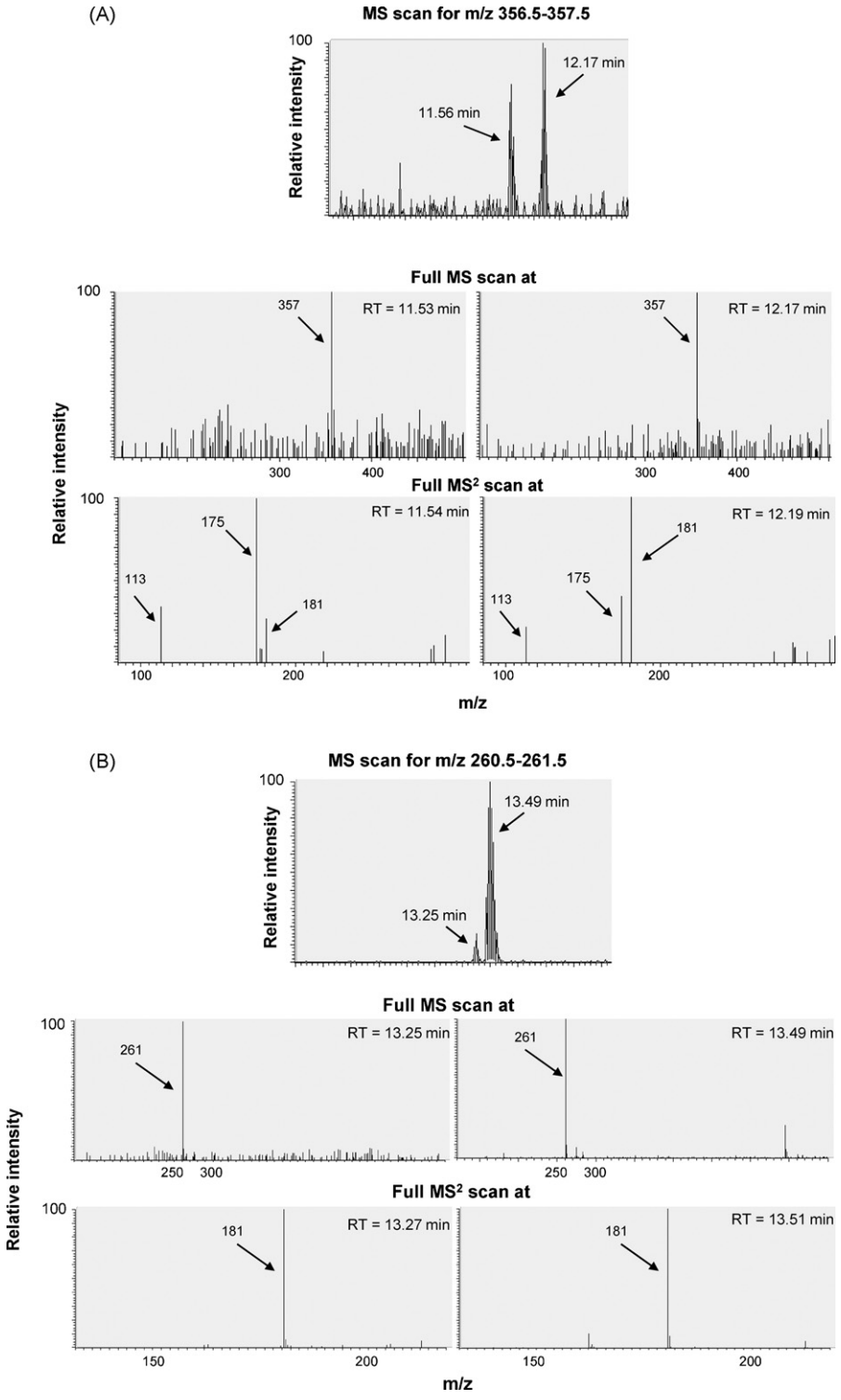

Fig. 2 – Chromatograms of plasma samples collected 30 min after oral administration of dihydrocaffeic acid. Dihydrocaffeic acid (100 μmol/kg) was orally administered to rats. Plasma was collected after 30 min, liquid–liquid extracted and analysed by LC–MS. MS^1 and MS^2 analyses were performed looking for the $[M-H]^-$ ions at *m/z* in the range of 356.5–357.5 (A) and 260.5–261.5 (B) corresponding respectively to the expected *m/z* of the glucuronide and sulphate conjugates of dihydrocaffeic acid. The arrows indicate the presence of the peaks of interest.

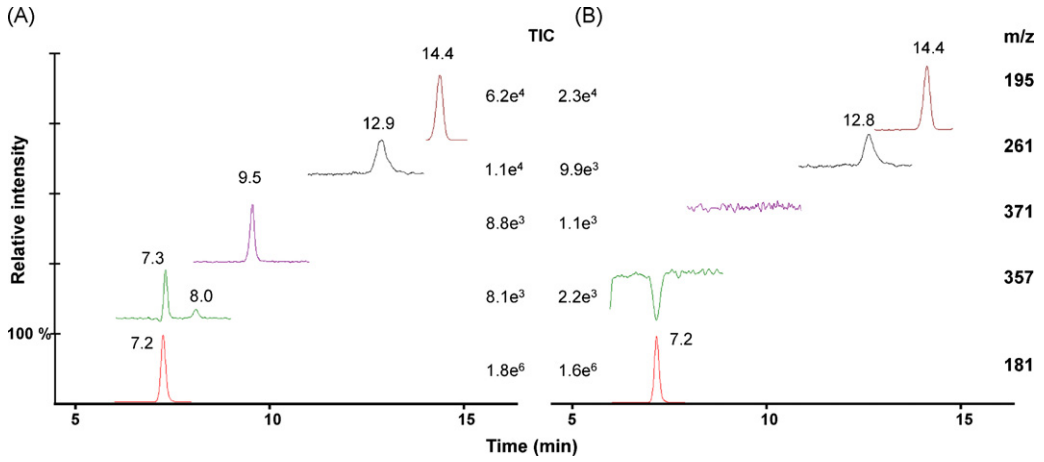

Fig. 3 – Chromatograms of the culture supernatants of HT29-MTX and Caco-2 cells incubated with dihydrocaffeic acid. HT29-MTX (A) and Caco-2 (B) cells were separately incubated with dihydrocaffeic acid (100 μM) for 3 h and supernatants analysed by LC–MS in the negative ion mode, searching for different mass to charge ratios. The detected peaks were identified as dihydrocaffeic acid (181), glucuronide of dihydrocaffeic acid (357), glucuronide of dihydroferulic acid (371), sulphate of dihydrocaffeic acid (261) and dihydroferulic acid (195).

3.2. *In vitro metabolism of dihydrocaffeic acid by the colonic epithelium*

In a recent paper, we reported that hydroxycinnamic acids, including dihydrocaffeic acid, are mainly passively transported by transcellular diffusion through the colonic epithelium [45]. However, the capacity of intestinal cells to metabolize dihydrocaffeic acid was not investigated at that time. Caco-2 (enterocyte-like) and HT29-MTX (globlet-like) cells were separately grown on dishes in order to identify metabolites produced from dihydrocaffeic acid (100 μM). LC–MS analysis of the HT29-MTX culture supernatants (Fig. 3A) revealed the presence of two peaks for the *m/z* at 357 (dihydrocaffeoyl-glucuronide), one peak for the *m/z* at 371 (dihydroferuloyl-glucuronide), one broad peak for the *m/z* at 261 (dihydrocaffeoyl-sulfate) and one peak for the *m/z* at 195 (dihydroferulic acid). No other compounds could be detected. A slightly different profile of metabolism was observed with Caco-2 alone since the glucuronides were absent from their supernatant (Fig. 3B), as already observed for ferulic acid [45]. The use of dihydrocaffeic acid glucuronide standards clearly confirmed peaks as dihydrocaffeoyl-4-O-β-D-glucuronide (RT = 7.3 min) and dihydrocaffeoyl-3-O-β-D-glucuronide (RT = 8.0 min). Multiple reaction monitoring (MRM), fragmenting the parent compounds and looking for pairs of parent > daughter ions, revealed the presence of two resolved but close peaks for the pair of *m/z* 261 > 181 (daughter > parent ions), at RT = 12.1 and 12.8 min. These peaks were unresolved by simple MS^1 analysis, the first one being much smaller than the second one and indicating that one of the hydroxyl groups may be favoured for sulphation. Only one methyl conjugate of dihydrocaffeic acid was detected, suggesting a preference for the methylation on one of the hydroxyl groups. However, caffeic acid, which also possesses two hydroxyl groups on the phenyl ring, when incubated with HT29-MTX and Caco-2 cells, was methylated into ferulic and isoferulic acid in not significantly different amounts ($P > 0.05$), indicating that the preference for methylation is substrate-dependent. The identity of the ion with *m/z* at 371 was confirmed as dihydroferuloyl-glucuronide by incubating the cultures directly with dihydroferulic acid which they were able to glucuronidate.

Transport and metabolism of dihydrocaffeic acid (1 mM) was also studied using the co-cultures of Caco-2 and HT29-MTX cells grown together on semi-permeable membranes as a model for the colonic epithelium. The main compound detected in the basal chamber was the parent compound dihydrocaffeic acid. The permeation rate of dihydrocaffeic acid was 1.9 ± 0.2 pmol/cm^2 min, $0.5 \pm 0.1\%$ of the initial quantity being transported over 1 h. Dihydroferulic acid, above the limit of quantification, was effluxed to the apical and basal sides at rates which were not significantly different, respectively 0.65 ± 0.16 and 0.50 ± 0.06 pmol/cm^2 min ($P > 0.05$).

3.3. *Ex vivo study of dihydrocaffeic acid transport and metabolism using everted sacs*

In order to support the results obtained *in vitro*, transport and metabolism of dihydrocaffeic acid were studied using an *ex vivo* model of rat colonic epithelium consisting of everted sacs of ascending and descending colon and the results were compared with data obtained with a segment of jejunum. The main compound transported to the serosal compartment was free dihydrocaffeic acid. Table 1 shows that the amount of dihydrocaffeic acid transported to the serosal side of the jejunal epithelium was not significantly different from the amount transported by the colonic epithelium. In parallel with its transport as the free form, dihydrocaffeic acid was also metabolized, as seen *in vitro*, and the metabolites effluxed to the serosal side of the epithelium. Dihydrocaffeic acid was conjugated with glucuronide or sulphate, but also methylated into dihydroferulic acid, which was itself found as glucuronidated and sulphated forms. The identity of the different metabolites was confirmed by MRM (Fig. 4) and by using the

Table 1 – Rate of dihydrocaffeic acid transport to the serosal side of everted sacs[a]

	Transport (pmol/cm^2 min)
Jejunum	38.8 ± 18.5
Ascending colon	58.2 ± 25.7
Descending colon	60.2 ± 31.4

[a] Everted sacs were incubated with dihydrocaffeic acid (500 μM) for 30 min at 37 °C, serosal side buffer extracted and analysed by LC–MS, with detection of the $[M-H]^-$ ion at *m/z* 181. The values are the mean ± S.D.

Table 2 – Efflux of dihydrocaffeoyl-glucuronides to the serosal side of everted sacs[a]

	Efflux of glucuronides (pmol/cm^2 min)	
	Gluc-4[b]	Gluc-3[b]
Jejunum	4.9 ± 7.3	32.2 ± 7.7
Ascending colon	22.4 ± 8.3[c]	101.9 ± 31.2[c]
Descending colon	50.8 ± 18.8[c]	203.2 ± 84.4[c]

[a] Everted sacs were incubated with dihydrocaffeic acid (500 μM) for 30 min at 37 °C, serosal side buffer extracted and analysed by LC–MS, with detection of the $[M-H]^-$ ion at *m/z* 357. The values are the mean ± S.D.
[b] Gluc-3 and Gluc-4: glucuronidation respectively on the 3- and 4-OH.
[c] Significantly different from the jejunum and Gluc-3 significantly different from Gluc-4 glucuronide ($P < 0.05$).

corresponding standards if available. As shown *in vitro*, the first peak obtained for *m/z* 357 (RT = 7.2 min) was dihydrocaffeoyl-4-*O*-β-D-glucuronide, the other one being its isomer, dihydrocaffeoyl-3-*O*-β-D-glucuronide. Only one large peak was apparent by MS^1 analysis for dihydrocaffeoyl-sulphate, but two peaks were resolved by MS^2, suggesting that dihydrocaffeic acid can be sulphated on one or the other hydroxyl groups of the phenyl ring, with a preference for one hydroxyl group, as proposed from the *in vitro* work reported above. Dihydroferulic acid, but not its isomer dihydroisoferulic acid, was detected. Dihydroferulic acid was itself further sulphated (*m/z* 275) and glucuronidated (*m/z* 371), possibly on the remaining free hydroxyl group on position 4. Other metabolites of dihydrocaffeic acid were looked for, but were absent, such as glycine (*m/z* 238) and mixed sulfo-glucuronide conjugates (*m/z* 437). Synthesized standards of dihydrocaffeoyl-glucuronides allowed quantification and showed a higher rate of glucuronidation and/or efflux by the colon compared with the jejunum (Table 2). Dihydrocaffeoyl-4-*O*-β-D-glucuronide was only detected in some everted jejunum samples in contrast to its isomer (3-form), which was the major form of glucuronide found in the serosal content of all the segments ($P < 0.05$). Whereas no difference was seen for dihydrocaffeic acid permeation through the epithelium of the different segments, dihydroferulic acid efflux by the colonic epithelium was significantly higher than by the jejunal epithelium (Table 3), indicating that the rat colon displays a higher catechol-*O*-methyl transferase activity for dihydrocaffeic acid compared with the small intestine. Moreover, it is interesting to notice that dihydrocaffeoyl-3-*O*-β-D-glucuronide was effluxed in significantly higher amounts than dihydroferulic acid ($P < 0.05$), suggesting that the main metabolism

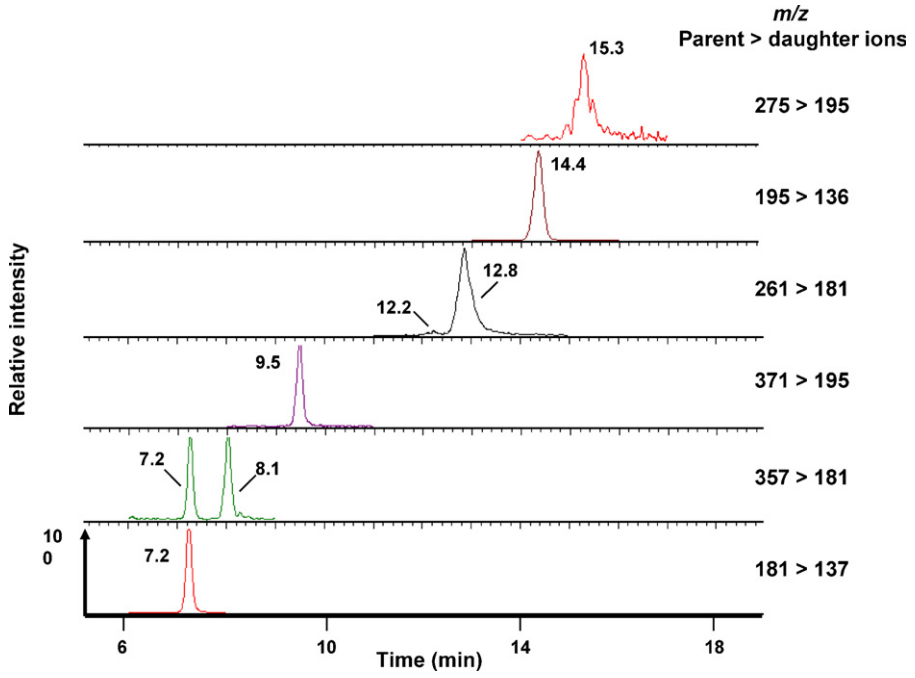

Fig. 4 – Chromatogram of an everted ascending colon content (serosal side) analysed by MS^2. Everted sacs were incubated with dihydrocaffeic acid (500 μM) for 30 min at 37 °C and their content analyzed by LC–MS looking for pairs of parent and daughter $[M-H]^-$ ions: 181 > 137 (dihydrocaffeic acid > parent minus –COOH), 357 > 181 (glucuronide > free dihydrocaffeic acid), 371 > 195 (glucuronide > free dihydroferulic acid), 261 > 181 (sulphate > free dihydrocaffeic acid), 195 > 136 (dihydroferulic acid > parent minus –COOH), 275 > 195 (sulphate > free dihydroferulic acid).

Table 3 – Efflux of dihydroferulic acid to the serosal side of everted sacs[a]

	Efflux (pmol/cm^2 min)
Jejunum	8.1 ± 2.2
Ascending colon	21.1 ± 5.6[b]
Descending colon	26.6 ± 8.9[b]

[a] Everted sacs were incubated with dihydrocaffeic acid (500 μM) for 30 min at 37 °C, serosal side buffer extracted and analysed by LC–MS, with detection of the $[M-H]^-$ ion at *m/z* 195. The values are the mean ± S.D.
[b] Significantly different from dihydroferulic acid present in the jejunum sac ($P < 0.05$).

performed by intestinal cells on dihydrocaffeic acid is the glucuronidation, followed by the methylation and then the sulphation.

3.4. *Ex vivo metabolism of dihydrocaffeic acid by the liver*

Even though not detected in the samples collected, dihydrocaffeic acid must be present at some point as the free form in plasma after crossing the gastric and/or intestinal epithelium, according to the results obtained with the *in vitro* and *ex vivo* models for the colonic epithelium. However, it must be rapidly cleared from plasma by further metabolism in the liver and excretion in urine. Therefore, the capacity of the liver to metabolize dihydrocaffeic acid was of interest. Metabolism of dihydrocaffeic acid, as well as of ferulic acid, was firstly studied by using HepG2 cells, the best characterized human hepatoma cell line. They express a variety of enzymes involved in the metabolism of xenobiotics by the liver, such as SULT activity, but these are passage-dependent and are expressed at low levels, although usually enough to study biotransformation of drugs [23]. We tried several culture and treatment conditions, but metabolites of dihydocaffeic acid could not be detected using the HepG2 cell model. We then tried an *ex vivo* model of liver slices reported to allow the total metabolism of 7-ethoxycoumarin in a time dependent manner for at least 10 h, longer than the dynamic organ system [24]. In order to stabilize dihydrocaffeic acid for the long incubation periods in culture medium, ascorbic acid (50 μM) was added to dihydrocaffeic acid (50 μM), which was as a consequence stable at 100% over 8 h of incubation. No peak was detected by searching the mass to charge ratio corresponding to the glucuronide (*m/z* 357) or the glycine (*m/z* 238) conjugates. However, two resolved peaks were detected for the *m/z* at 261, corresponding to two forms of dihydrocaffeoyl-sulphate and supporting the idea that dihydrocaffeic acid can be sulphated on either the 3 or the 4 hydroxyl groups of the phenyl ring (Fig. 5). Dihydroferulic acid (*m/z* 195) and its sulphate conjugate (*m/z* 275) were also found, but only one peak was detected for each mass to charge ratio, indicating that neither dihydroisoferulic acid nor its sulphate could be detected. Dihydroferulic acid was detected after 30 min of incubation of dihydrocaffeic acid with the liver slices, and was quantifiable after 2 h. The amount of dihydroferulic acid produced after 8 h was 2.12 ± 0.33 nmol/mg protein ($n = 3$), corresponding to 5.3 ± 0.8% of the initial concentration of dihydrocaffeic acid. Moreover, two peaks were found at *m/z* 193, the first and major one (RT = 22.25 min) being ferulic acid and the second one (RT = 23.3 min) being isoferulic acid. From these results, we hypothesize that the first and major peak found for *m/z* 273 could be feruloyl-sulphate whereas the second and minor one could be isoferuloyl-sulphate. Ferulic acid concentration increased over time to reach 0.37 ± 0.11 nmol/mg protein

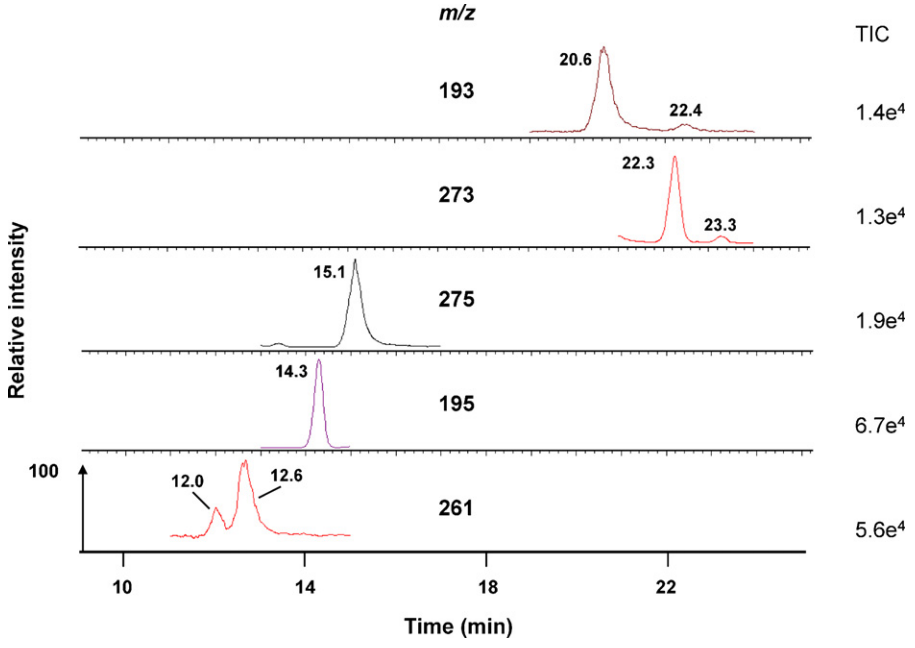

Fig. 5 – Chromatogram of the supernatant of liver slices cultures analysed by LC–MS. Supernatants of liver slice cultures were collected after 8 h incubation with dihydrocaffeic acid (50 μM) in the culture medium. Supernatants were analyzed by single ion recording of the $[M-H]^-$ ions with mass to charge ratio: 181 (dihydrocaffeic acid), 261 (sulphate of dihydrocaffeic acid), 195 (dihydroferulic acid), 275 (sulphate of dihydroferulic acid), 273 (sulphate of ferulic acid) and 193 (ferulic acid).

(0.9 ± 0.3% of the initial quantity of dihydrocaffeic acid). Interestingly, no caffeic acid (*m/z* 179) could be detected in the supernatant of the liver slice cultures. This finding supports the hypothesis that, in the cascade of dihydrocaffeic acid metabolism, ferulic and isoferulic acids must respectively result from the oxidation of the side chain of dihydroferulic acid and its isomer, rather than from a methylation of one or the other hydroxyl groups of caffeic acid. However, caffeic acid can be methylated into ferulic and isoferulic acid by intestinal cells, as seen using the *in vitro* model for colonic epithelium (data not shown). In order to confirm that dihydroferulic acid was the source of ferulic acid from dihydrocaffeic acid, we incubated dihydroferulic acid with liver slices. Ferulic acid was detected after 30 min of incubation and was quantifiable after 2 h, its concentration increasing over time and reaching 8.0 ± 1.8% of the initial amount of dihydroferulic acid (50 μM) after 8 h. Interestingly, liver slices were also able to reduce the double bond of the side chain leading to the production of dihydroferulic acid from ferulic acid. However, no isoferulic acid could be detected from dihydroferulic acid, supporting the idea of the methylation on the 4-hydroxyl group of dihydrocaffeic acid as the route to isoferulic acid production, rather than a de-methylation and re-methylation on the other hydroxyl group. Liver slices mainly produced the sulphated form of ferulic acid, followed by its reduced form (2.2 ± 0.4% of the initial concentration of ferulic acid). However, ferulic acid was glucuronidated by the liver although no glucuronide of dihydrocaffeic and dihydroferulic acids could be detected.

4. Discussion

Fig. 6 summarizes our findings on the metabolic fate of ingested dihydrocaffeic acid. After gavage to rats, dihydrocaffeic acid was very rapidly absorbed and cleared from the plasma, since it was only found unconjugated in the urine and not in blood. This suggests that dihydrocaffeic acid absorption must have taken place in the stomach and/or in the upper part of the small intestine. In addition to transport, it is likely that gastric cells could also metabolize part of the dihydrocaffeic acid absorbed, since absorption from the stomach has already been proposed for flavonoids [25,26] and phenolic acids [27,28]. However, the first study reporting ferulic acid absorption by the stomach suggested only further metabolism by the liver [28]. The second report mentioned a possible metabolism of ferulic acid in parallel to its absorption by the gastric cells, since conjugates were detected in plasma from the portal vein only 5 min after the intake. However, taking into account their previous findings, the authors came to the conclusion that there was rather a metabolism of ferulic acid by the liver and/or re-absorption by the entero-hepatic circulation than a

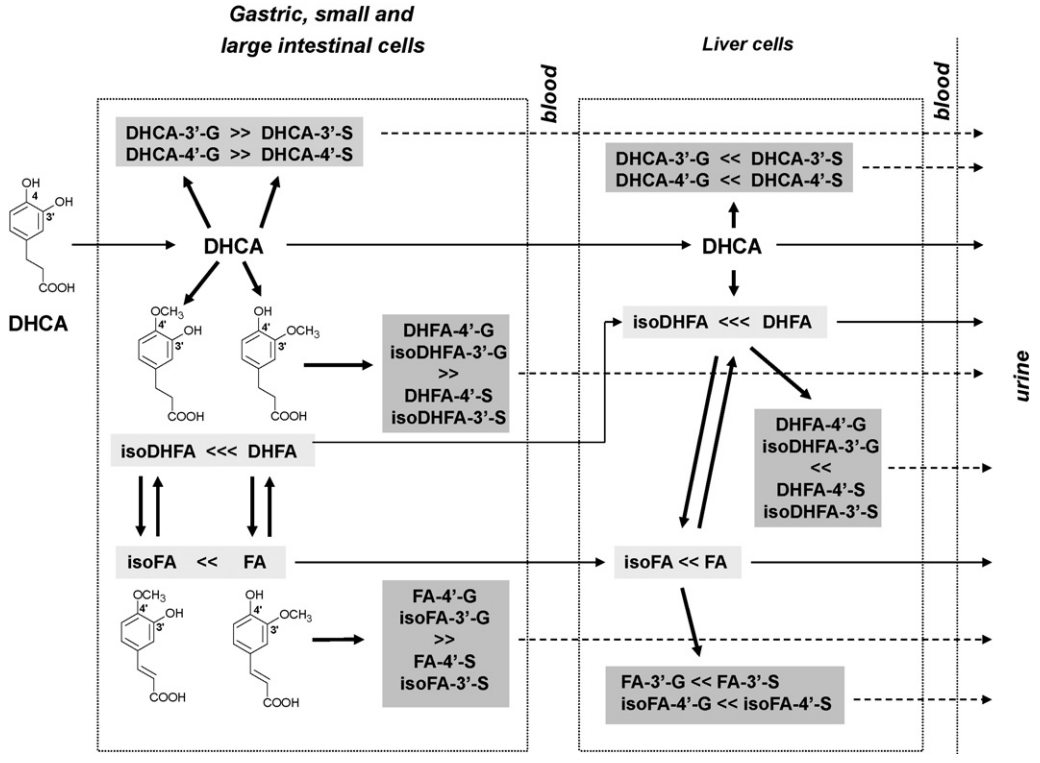

Fig. 6 – Proposed metabolic fate of dihydrocaffeic acid in rats. Depending on the food source or delivery, dihydrocaffeic acid may be absorbed by: the stomach or the duodenal or jejunal cells, if given as the free form; or rather by the ileum or the colon after metabolism by the microflora, if given as an esterified form (e.g. as present in many foods) or if given as a precursor (e.g. catechins). The unbroken arrows indicate that the permeation or efflux may be by transcellular diffusion and, to a smaller extent, by facilitated transport. The dotted arrows represent a potential facilitated transport. DHCA, dihydrocaffeic acid; (iso)DHFA, dihydro(iso)ferulic acid; (iso)FA, (iso)ferulic acid. 3′ or 4′-G/S, glucuronidation or sulphation on the 3′- or 4′-OH.

metabolism by the stomach [27]. The presence of UDP-glucuronosyltransferases, as well as sulfotransferases, has been detected in stomach [29–31], indicating that gastric cells could be involved in a pre-systemic detoxification of compounds absorbed by the stomach, even before reaching the liver. The transport of dihydrocaffeic acid to the serosal side of the gastro-intestinal tract was supported by the results obtained with the *in vitro* and *ex vivo* models for the colonic epithelium, which were able to transport but also to glucuronidate and sulfate the hydroxycinnamic acid. In contrast to the HT29-MTX cells, the Caco-2 cells do not appear to express a UDP-glucuronosyltransferase activity towards dihydrocaffeic acid as shown for epicatechin [32], even though able to glucuronidate different flavones [33] and resveratrol [34]. Moreover, Kern et al. reported the production of glucuronides by Caco-2 cells from methyl-ferulate, -sinapate, -caffeate and -*p*-coumarate but not from the corresponding free acids [35]. Taken together, all these findings suggest that different UGTs must have different specificities for substrates and that the isoform involved in the glucuronidation of dihydrocaffeic acid must be absent from Caco-2 cells. In addition, metabolism of dihydrocaffeic acid using rat intestine suggests that the UDP-glucuronosyltransferase activity, favouring glucuronidation of the 3-OH over the 4-OH, is higher in the colonic than in the jejunal epithelium and is almost absent in the rat liver. Even though relatively less expressed in the lower part (caecum, colon, rectum) than in the upper part (duodenum, jejunum, ileum) of the rat gastrointestinal tract, UGT1A7 expression is higher in the colon than in the liver [29,36]. Interestingly, transcripts of the isoform UGT1A7 have not been detected in Caco-2 cells [37] whereas the gene is expressed by HT29 cells [38], the precursor of the HT29-MTX cells. Moreover, UGT1A7 is ubiquitously expressed in human with trace to low levels in the gastrointestinal tract [39], being more abundant in the stomach [37]. All these findings would suggest that the isoform UGT1A7 is a potential candidate for the glucuronidation of dihydrocaffeic acid, and probably of other hydroxycinnamic acids, observed with HT29-MTX cells and with the gastrointestinal tissue of rats. We could hypothesize that if the UGT1A7 is the only isoform involved in the glucuronidation of dihydrocaffeic acid, in humans this conjugation would occur mainly in the stomach but at much lower levels, or not at all, in the lower part of the gastrointestinal tract and in the liver. However, these hypotheses do not exclude that, rather than an absence of a specific UDP-glucuronosyltransferase activity for dihydrocaffeic acid in Caco-2 cells, there could be a deficiency in the efflux system of the glucuronide in these cells compared with HT29-MTX cells and that this efflux mechanism would be more expressed in the colon than in the jejunum of the rat. An opposite profile of activity has been shown for the sulphation of dihydrocaffeic acid compared with glucuronidation, which was favoured in the liver compared with the intestine of rat. Caco-2 have also been shown to express SULT1A1 and SULT1A3 at identical levels [40] and are able to produce sulphate conjugates of epicatechin [32], indicating that the SULT activity is present in the Caco-2 cells. In rats, the highest amount of SULT mRNAs is detected in the liver with significant expression of SULT1A1 and SULT1B1, the latter being more expressed than the former in the intestine [41]. In humans, SULT1A1, A3 and B1 are expressed in all the gastrointestinal tract, as seen by immunohistochemistry and immunoblotting, the levels being the highest in the ileum, and SULT1A1 was more expressed in the ileum than the liver [31]. SULT1A3, higher than SULT1A1, is absent in the liver and SULT1B1 is 10 times higher in the ileum than in the liver. According to all these findings, tissue distribution of SULT activity would be different in human and in rats, in humans the gastrointestinal tract being more potent whereas in rats it would be the liver.

Dihydrocaffeic acid, in addition to glucuronidation and sulphation, was also methylated but only one form was detected and was identified as dihydroferulic acid. We have previously shown that the mass spectral signal from phenolic acids with a 3-hydroxyl (such as isoferulic acid) can be severely suppressed when the HPLC solvent contains an acid modifier to ensure good chromatography [42]. The LC–MS conditions used in this study were adjusted so as to ensure that isoferulic acid could be detected, albeit with a lower sensitivity than for ferulic acid. Because we did not have a standard of dihydroisoferulic acid it has not been possible to investigate the susceptibility of this compound to ion suppression during LC–MS, but we assume that it would be very similar to isoferulic acid, and it is possible that if only a small amount of dihydroisoferulic acid was produced during metabolism, then its presence may not have been detected. Moreover, only one sulphated or glucuronidated form of dihydroferulic acid could be detected. The ion suppression is not expected to impair the detection of conjugated forms, suggesting that if the conjugates of dihydroisoferulic acid were present in the samples in amount above the limit of detection, we would have seen them on the chromatograms. The lack of detection of dihydroisoferulic acid was more due to low production, under the limit of detection, rather than to ion suppression. Nevertheless, it is hypothesized that both methylation, on the 3- and 4-hydroxyl groups of dihydrocaffeic acid, must occur since 2 compounds, ferulic and isoferulic acid, appear after ingestion of dihydrocaffeic acid *in vivo* or when incubated with colonic epithelium or liver slices, although one being favoured over the other. These data support the fact that catechol-O-methyl-transferase (COMT) strongly favoured the 3- compared with the 4-hydroxyl group of dihydrocaffeic acid for methylation, leading to the formation of 3-methoxy-4-hydroxy-phenylpropionic acid (dihyroferulic acid). A study on understanding of the COMT regio-selectivity towards the hydroxyl groups of norepinephrine [43] suggested that there is a link between the pK_a of the hydroxyl groups and the regio-selectivity for methylation of norepinephrine by the COMT. According to the authors, the 4-O-methylation requires a pre-ionization of the hydroxyl group, whereas the 3-O-methylation is assisted by a base present in the COMT allowing methylation to occur at lower pH than the pK_a. Whereas only one form of methyl conjugate was detected from dihydrocaffeic acid, caffeic acid was metabolized into ferulic and isoferulic acids in similar amounts, indicating that there was not as much regio-selectivity for caffeic acid methylation as there was for dihydrocaffeic acid. These data support the hypothesis of pK_a-dependent methylation of the 4-OH. Indeed, caffeic and dihydrocaffeic acids both possess a 3-OH pK_a of 11.38 ± 0.02 whereas the 4-OH of caffeic acid at 8.48 ± 0.05 is closer to neutral pH than is the one of dihydrocaffeic acid

(9.24 ± 0.02) [17] and as a consequence, more liable for methylation.

Dihydrocaffeic and caffeic acids were methylated respectively into dihydroferulic and ferulic acids by intestinal and hepatic cells. However, the reverse reaction was not observed in contrast to what was shown previously using CYP1A1/2 induced liver microsomes and a 10 times higher substrate concentration than used here [44]. This conversion may have happened in our model but the amount of the resulting compounds must have been under the limit of detection, suggesting that, *in vivo*, de-methylation must be a secondary metabolic pathway, the expected aim of the liver being to detoxify by neutralizing hydroxyl functions rather than liberating them. However, hepatic and intestinal cells were also able to reduce ferulic acid into dihydroferulic acid, and catalyse the reverse reaction, but no interconversion between dihydrocaffeic and caffeic acids was detected in contrast to reports by Moridani et al. using isolated hepatocytes [44].

In summary, the results indicate that dihydrocaffeic acid is absorbed and extensively metabolized, although some free form is found in urine. Our findings suggest that rat intestinal epithelium favours the glucuronidation of dihydrocaffeic acid, whereas the rat liver favours sulphation, both the conjugations and methylation favouring the 3-OH position (summarised in Fig. 6). The metabolic profile proposed in the present report is based on results found with rat tissues. This profile could be different in other species, and especially in human, due to probable different rates of expression and/or tissue distribution of the metabolizing enzymes. However, the metabolism of dihydrocaffeic acid obtained with the human intestinal cells (HT29-MTX) also suggests a possible higher rate of glucuronidation than sulphation in the human intestine. The results show the pathways of absorption and metabolism of dihydrocaffeic acid, an important bioactive substance, and will facilitate future *in vivo* studies in humans.

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